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Characterisation of Scp1p, an actin-bundling protein in the budding yeast *Saccharomyces cerevisiae*

By

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A thesis submitted to the
Faculty of Biomedical and Life Sciences
for the degree of
MASTER OF SCIENCE



UNIVERSITY
of
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Abbreviations

2xYT	Yeast -Tryptone medium
ABD	Actin Binding Domain
ABS	Actin Binding Site
CD	Circular Dichroism
CH domain	Calponin Homology
CLR/CLIK23	Calponin Like Repeats
EM	Electron Microscopy
F-actin	Filamentous actin
G-actin	Globular actin
KME	KCl-MgCl ₂ -EDTA solution
MS	Mass Spectrometry
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
SDS-PAGE	SDS-Poly-Acrylamide Gel Electrophoresis
TBST	Tris-Buffered-Saline-Tween solution
TDE	Tris-Deoxycholate-EDTA solution
TDEN	Tris-Deoxycholate-EDTA-NaCl solution
TE	Tris-EDTA solution
YPAD	Yeast extract-Peptone-Adenine-Dextrose medium

Abstract

The organisation of the actin cytoskeleton within eukaryotic cells is controlled by a large variety of actin-binding proteins (ABPs) with roles ranging from nucleation to bundling, severing and capping of actin filaments. The calponins and transgelins are a family of actin-binding/bundling proteins possessing one or more highly conserved motifs (i) calponin homology (CH) domain, and (ii) calponin-like repeat (CLR/ CLIK23 repeat), which have both been implicated in actin-binding. The *Saccharomyces cerevisiae* gene SCP1 encodes a protein, which shares a high degree of homology with SM22/transgelin. In this study Scp1p was purified and, using a range of biochemical techniques was shown to possess actin-bundling activity. Further analysis by EM revealed the formation of tight bundles of actin in the presence of Scp1p.

Although a large number of protein containing CH domains have been shown to bind actin, the CH domain alone is not sufficient to bind actin. Using amino and carboxy terminal (N- and C-term) Scp1p truncated mutants it has been possible to localise the actin-binding domain (ABD) to the C-terminal region containing the CLR/CLIK23 repeat, and to show that the CH domain alone is not able to bind actin.

In vivo studies into the localisation of Scp1p have revealed a co-localisation with actin to patches at the cell cortex. Other proteins found at these actin patches include the yeast fimbrin homologue Sac6p, another CH domain-containing protein involved in actin bundling. It has been shown that deletion of the SCP1 gene alone has no adverse affect on actin organisation, whereas a SAC6/SCP1 double knockout displays a more defective actin phenotype than the SAC6 single knockout. The importance of Scp1p in organising the actin cytoskeleton is clearly shown in cells overexpressing Scp1p, which possess a severely disrupted actin cytoskeleton, with the appearance of large clumpy actin patches. Taken together this study shows that Scp1p is an actin-bundling protein, which is involved with Sac6p in the organisation of the yeast cytoskeleton.

1. Introduction

1.1 The eukaryotic cytoskeleton

The components of the cytoskeleton and the proteins that help regulate it are highly conserved and make up a large amount of the total protein in eukaryotic cells. The cytoskeleton represents a three dimensional meshwork of interconnected proteins that (i) controls the shape of the cell, (ii) co-ordinates and drives the movement of the cell, and (iii) facilitates the intracellular movement of organelles and other proteins. It consists of three main filamentous networks:- (i) microtubules (ii) microfilaments, and (iii) intermediate filaments, with each being comprised of different proteins. These networks will be discussed in turn.

1.1.1 Microtubules

Microtubules are the largest of the three filaments and are made of tubulin, which is a 55 kDa protein found as both α , and β forms. It actively forms heterodimers, and it is these which assemble in the presence of Mg^{2+} and GTP to form microtubules. These structures are made from parallel bundles of thirteen protofilaments and appear as hollow tubes 25 nm in diameter. They exhibit polarised growth with one end being able to polymerise tubulin three times quicker than the other. Nucleation occurs at the centrosomes, which are the major microtubule organising centres (MTOC) in most animal cells and extend towards the cell periphery. Microtubule associated proteins (MAPs) such as dynein and kinesin utilise the microtubule network in the transport of organelles around the cell. It is worth noting that in *Saccharomyces cerevisiae* the role of intracellular transport falls on microfilaments, with microtubules being involved in the nuclear movement to the bud site and chromosome segregation.

1.1.2 Intermediate filaments

Intermediate filaments (IFs), which are not found in all cells types, are made up of a variety of different fibrous proteins, including keratins, desmin, nuclear lamins and vimentin. These proteins are rigid structures with a diameter of around 10 nm found mainly in cells that are under mechanical stress (eg epithelia, nerve axons) and are the most abundant proteins in skin and hair. The various proteins which can form IFs have similar domain organisation, namely an amino terminal head domain, a central α helical rod domain and a carboxy terminal tail domain. They appear to be limited in function to one of providing tensile strength to certain cells and tissues, with relatively little being known about the diversity of the head and tail regions and their possible roles (Strelkov, Herrmann, & Aebi 2003)

1.1.3 Microfilaments

The third type of filaments are the microfilaments; these are made of the polymers of the 43 kDa protein actin and appear as threadlike structures about 8 nm in diameter. Polymerisation of actin requires both monovalent and divalent cations as well as ATP. Microfilaments are highly dynamic structures and are regulated by a wide variety of actin-binding proteins. They help to provide shape to cells, and are connected to the cellular membranes by membrane microfilament-binding proteins. In most cells microfilaments exist as a dense mesh-like network below the plasma membrane called the cortex. Cellular locomotion occurs as a result of the polarised polymerisation of microfilaments at the leading edge of motile cells, and can be seen in the formation of filipodia, lamellipodia and membrane ruffles. It is also worth noting that in *S. cerevisiae* microfilaments confer polarity on the cell, with a rearrangement of their distribution occurring during the cell cycle.

1.2 Actin

Actin is one of the most abundant proteins found in eukaryotic cells, and can account for up to 5% of the total cellular protein in non-muscle cells. It is highly conserved

throughout all of the eukaryotes with less than 20% difference in sequence being observed between mammalian and yeast actin. It is expressed from one gene in yeast (ACT1) and from up to 60 in some plants (Meagher 1991), with the human genome possessing six actin genes. In humans there are three different types of actin; α , β and γ with four alpha isoforms found in muscle cells and the β and γ isoforms being found in non-muscle cells (Pollard, Blanchoin, & Mullins 2001). Actin is a globular protein (G-actin) in its monomeric form that polymerises to a filamentous form (F-actin) in a concentration-dependent manner.

Since all eukaryotic cells have an abundance of actin, with there being almost no difference in the structure of the actin isoforms it is intriguing that the actin cytoskeleton can play such diverse roles in different cell types. The answer to this problem lies in the large variety of actin-binding proteins (ABPs) that interact with different domains on the surface of the protein and act to control the occurrence or distribution of various actin structures within the cell's complement of proteins, (dos Remedios et al. 2003; McGough 1998; Otto 1994). Many of these ABPs are also controlled by a wide range of intracellular second messengers; resulting in a cytoskeleton that is highly dynamic in its re-organisation in response to both internal and external stimuli.

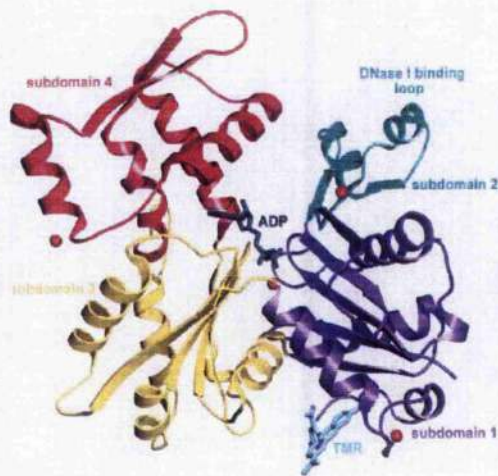
It is also of note that although actin is absent in prokaryotic cells, its ancestry may lie in the protein MreB that has recently been shown to form filamentous structures located close to the cell surface of *Bacillus subtilis* (Jones, Carballido-Lopez, & Errington 2001). Although the amino acid sequence of MreB has only 15% identity with actin, the structure of its filaments is remarkably similar though it forms only single rather than paired helices (van den, Amos, & Lowe 2001).

1.2.1 G-actin

G-actin is a single polypeptide chain of 375 amino acids and a molecular weight of 43 kDa; it was first predicted to exist as a pear-shaped molecule with a "large" and a "small" domain (Aebi et al. 1981).

Although the first crystals of monomeric actin were reported over 20 years ago when it was co-crystallised with pancreatic DNase1 (Mannherz, Kabsch, & Leverman

a.



b.

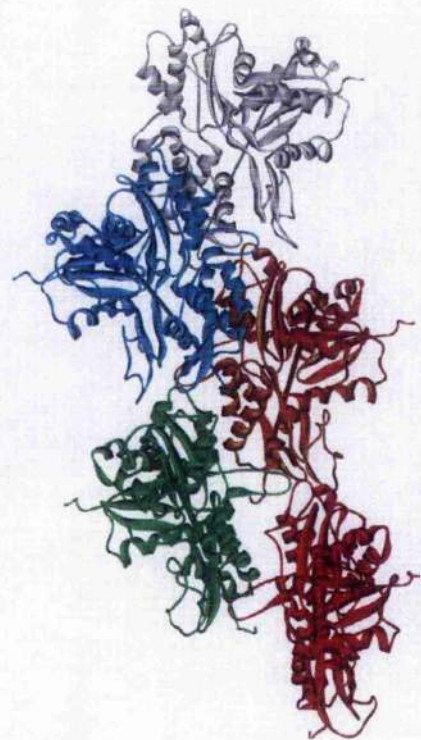


Figure1. 1 Actin Structure

(a.)Ribbon diagram showing G-actin in the ADP-bound state. It is comprised of 4 sub-domains labelled 1, 2, 3, and, 4; with a DNase1 binding loop situated at the top of sub-domain 2. Four Ca²⁺ ions are represented as gold spheres and can be seen bound to sub-domains 1,2, and 4, as well as in close proximity to the nucleotide binding cleft. Taken from Otterbein et al 2001(b.)The "Holmes model" of an actin filament in which we can see six actin monomers exhibiting a ¼ turn of a two start, right handed, long pitch helix. Taken from Dos Remedios et al 2003.

1977), it was not until 1990 that the structure was solved to a resolution of 2.4Å (Kabsch et al. 1990). Further modifications were made to this structure by the subsequent crystallisations firstly with gelsolin-1 (McLaughlin et al. 1993) and subsequently with profilin (Schutt et al. 1993). It was not until 2001 that crystals of monomeric actin not complexed with ABPs were produced (Otterbein, Graceffa, & Dominguez 2001). These studies have revealed a structure with a large α -helical content (Fig. 1.1a) that can be divided into four sub-domains numbered 1, 2, 3, and, 4 with both the amino and carboxy termini located at sub-domain 2. These sub-domains surround a cleft which is occupied by ATP or ADP, the former binding with an affinity ten times greater than that of the latter, and in complex with either Mg^{2+} (K_d 1.2nM) or Ca^{2+} (K_d 0.12 nM). Conformational changes which can cause an opening and closing of this nucleotide binding cleft (Tirion et al. 1995) have been ascribed to a “hinge” at residues 140 and 338, with other changes in conformations occurring as a result of actin binding to different ABPs (Dedova et al. 2002).

1.2.2 F-actin

Although the atomic structure of F-actin has yet to be solved, it has been long been known to exhibit a helical nature (Hanson & Lowy 1964), with a more accepted model of it showing a two-start, right handed, long pitch helix (Holmes et al. 1990) (Fig. 1.1b) comprising 12-14 monomers per half turn. Further refinements to this model have been made using actin mutants (Lorenz, Popp, & Holmes 1993) and also by taking into account the hinge motion of the domains at the nucleotide-binding cleft (Tirion, ben Avraham, Lorenz, & Holmes 1995).

The polarity of F-actin can be seen in electron micrographs of actin filaments decorated with myosin sub-domain 1 (Lewis & Bridgman 1992). The myosin binds to the side of the filaments with a slight tilt and gives the appearance of arrowheads (Fig. 1.2) with their “pointed” ends aligning with sub-domains 2 & 4, and sub-domains 1 & 3 being represented by the “barbed” ends.

In vitro polymerisation of G-actin to F-actin occurs in three main stages (Oosawa et al. 1977) and is promoted by high ionic strength and a slightly acidic or neutral pH



Figure 1.2 F-actin exhibits polarity

An electron micrograph showing myosin S1 binding to actin filaments exhibits polarity and gives the appearance of arrow heads, with the pointed ends (p) representing sub-domains 2 and 4; and the barbed ends (b) representing sub-domains 1 and 3. Taken from Lewis and Bridgeman 1992

(Greer & Schekman 1982a; 1982b). This transition is reversible with depolymerisation occurring when the ionic strength of the solution is lowered.

First there is the lag phase, which consists of a slow dimerisation step followed by the formation of a stable trimer known as the nucleus. Next follows a rapid elongation phase where G-actin can associate with both the barbed and pointed ends of filaments, although the former occurs at a rate 5-10 times faster than the latter. The third phase, known as the steady state, occurs when G-actin monomers are exchanged at both ends with no resulting change in the length of the filament. (Fig. 1.3).

In vivo the majority of G-actin subunits contain ATP within their nucleotide binding cleft; the subsequent polymerisation into F-actin results in the hydrolysis of this ATP to ADP followed by release of P_i (Carlier, Pantaloni, & Korn 1987). Originally this ATPase activity was thought to be necessary for actin polymerisation, although this has now been disproved by experiments using G-actin bound to ADP or non-hydrolysable analogues of ATP (Pollard & Weeds 1984). Once disassociated from a filament, ADP bound to actin is rapidly exchanged for ATP in solution (Neidl & Engel 1979) thus allowing the cycle to begin again.

1.2.3 The polarity of actin polymerisation/depolymerisation

The concentration above which actin polymerisation, and below which depolymerisation occurs, is known as the critical concentration (CC) and is found to be 12 to 15 fold higher at the pointed than at the barbed end (Wanger & Wegner 1983). When the G-actin concentration is lower than the CC of the barbed end, there is no polymerisation of the filament; when it is higher than the CC of the pointed end, polymerisation occurs at both ends. When the concentration falls between the CC of both ends polymerisation only occurs at the barbed end. Once a steady state is reached where the rate of polymerisation is equal to that of depolymerisation, a treadmilling effect is observed with actin subunits being sequentially added to the barbed end, moving along the length of the filament, and dissociating from the pointed end (Fig.1.3) (Pantaloni, Clainche, & Carlier 2001).

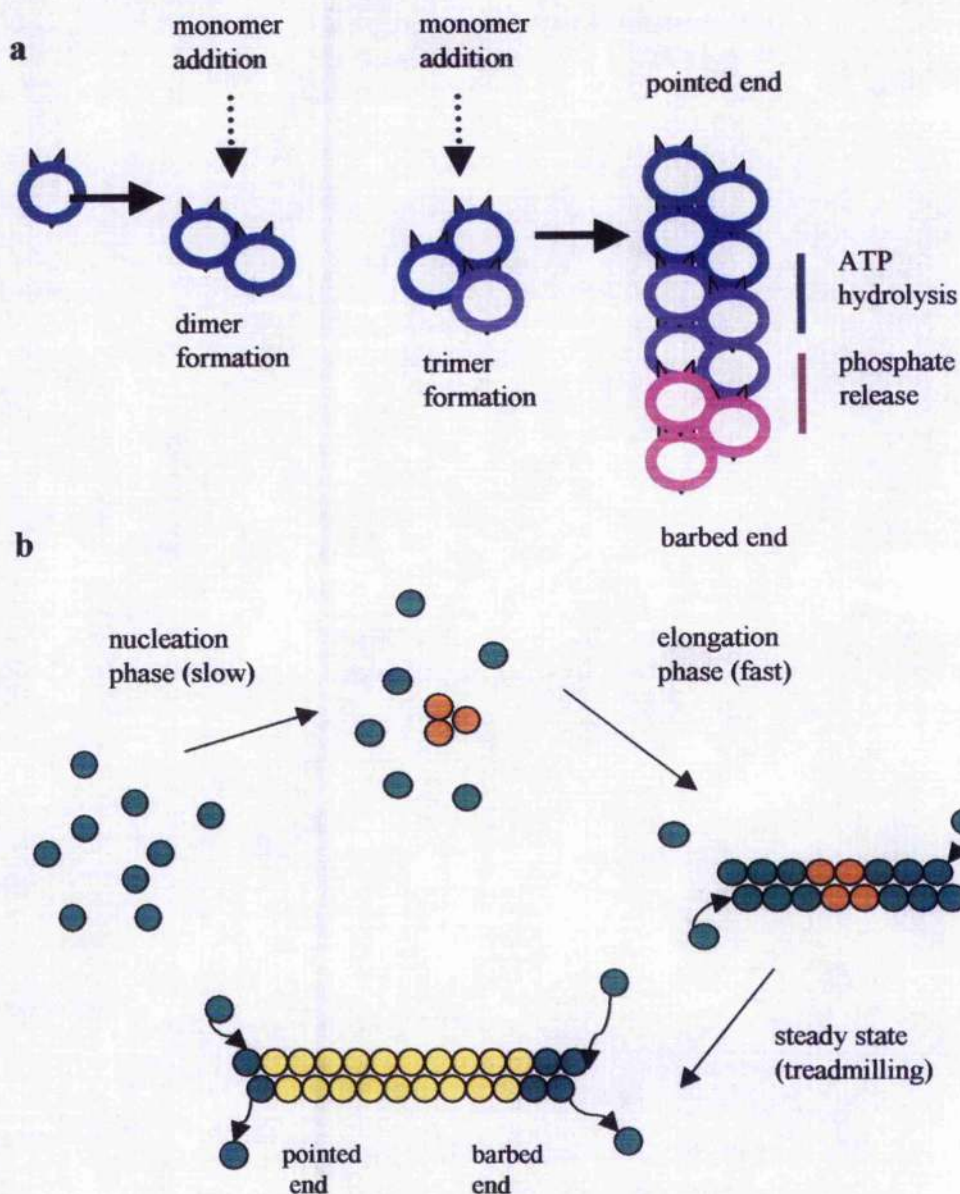


Figure 1.3 The polymerisation of actin monomers into actin filaments.

(a) During the initial nucleation phase, ATP-G-actin monomers (blue) slowly form stable trimeric complexes which are then more rapidly elongated in the second phase of polymerisation. After their incorporation into a filament, subunits slowly hydrolyse ATP to ADP and unreleased-phosphate(Pi) (purple). The 'oldest' or pointed end contains ADP-bound monomers from which the Pi has been released (pink). The actin filament displays polarity as G-actin adds primarily to the barbed end and loss occurs mainly at the pointed end. (b) There are three phases for *de novo* G-actin polymerisation *in vitro*; nucleation, elongation and steady state.

1.3 *Saccharomyces cerevisiae* as a model eukaryotic system

The structures of many proteins as well as their intra-cellular localisation and functions are highly conserved throughout all eukaryotic organisms. Consequently, studies using the budding yeast *S. cerevisiae* as a model system have provided valuable information towards the understanding of a wide range of biological events within these cells and those of higher eukaryotes (Ayscough & Drubin 1996).

S. cerevisiae is a unicellular organism that exists in either haploid or diploid states, and grows both in liquid broth and on a solid agar plate. It can be studied using biochemical or immunofluorescence techniques as well as by conventional genetics with new strains being easily generated by crossing parental strains with different genotypes. The sequencing of the 6000 genes of its genome has resulted in many new proteins being discovered, as well as allowing previously known proteins to be easily mutated using PCR and other molecular biology techniques

1.3.1 F-Actin structures in *S. cerevisiae*

The different actin structures can be visualised in yeast by fluorescence microscopy using either fluorescently labelled anti-actin antibodies or rhodamine-phalloidin, a fluorescently labelled phallotoxin that binds to F-actin (Adams & Pringle 1991). Using these techniques it is possible to discern two distinct actin structures, cytoplasmic actin cables and cortical actin patches (Kilmartin & Adams 1984). Both structures are dynamic with reorganisation occurring throughout the cell cycle (Fig. 1.4) (Pruyne & Bretscher 2000). Their location and orientation confer polarity on a cell (Drubin & Nelson 1996) and their interactions with a large number of associated ABPs is thought to control the organisation of the actin cytoskeleton (Ayscough & Drubin 1998; Schott, Huffaker, & Bretscher 2002; Warren et al. 2002)

1.3.2 Actin Patches

Cortical actin patches contain most of the actin in yeast and are highly motile structures (Doyle & Botstein 1996) containing more than 30 associated proteins

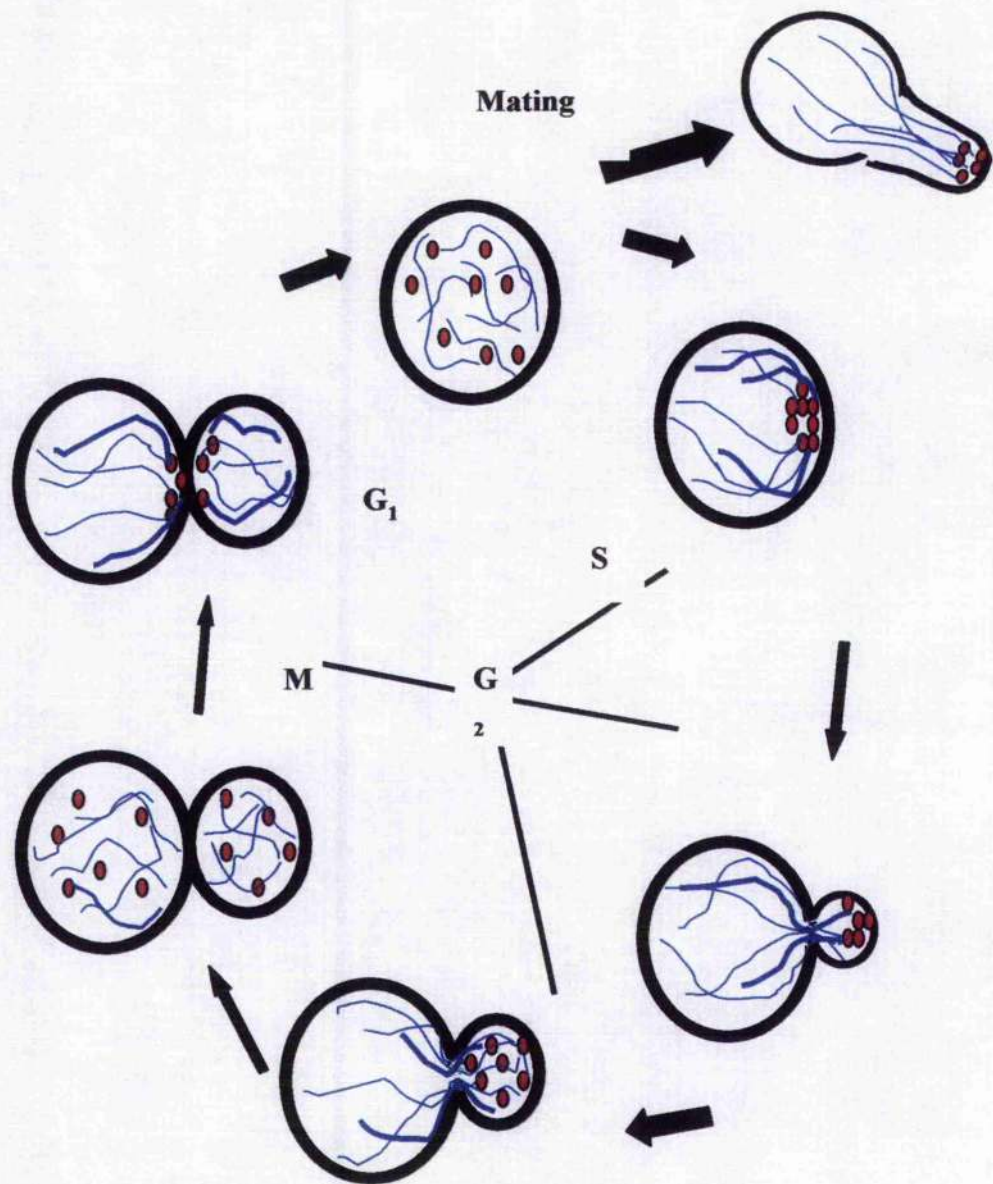


Figure 1. 4 Organisation of the actin cytoskeleton during vegetative growth and shmoo formation in budding yeast. Actin cables (blue) and cortical actin patches (red) show a distinctive polarised distribution throughout the cell cycle and during shmoo formation for mating. Cortical actin patches cluster at sites of active growth while the actin cables become arrayed parallel to the mother-bud axis to guide secretory vesicles and organelles to the sites of active growth. Adapted from Pruyne and Bretscher 2000

(Pruyne & Bretscher 2000). These include:- Arp2/3 complex (actin nucleating complex of 7 protein subunits), Las17/Bee1p (nucleation regulators), Sac6p (bundling protein), Cap1/2p (capping proteins), Pan1p (Eps15 homology), Sla1p and Abp1p. The patches are located at tubular plasma membrane invaginations (Mulholland et al. 1994) and are concentrated at sites of polarised growth in the yeast cell cycle (Adams & Pringle 1984). It is important to note that the motility of actin patches does not require the myosin motor proteins, but is instead reliant on the Arp2/3 complex indicating a role for actin polymerisation in motility (Cope et al. 1999).

The function of actin patches in the cell is still unclear, although they do appear to play an important role in secretion and in the internalisation step of endocytosis (Geli & Riezman 1998; Pruyn & Bretscher 2000). A model of this interaction (Warren, Andrews, Gourlay, & Ayscough 2002) utilises the Abp1p binding partner Sla1p as a scaffold protein which can link the actin patch components with the endocytic machinery through an interaction between the C-terminal repeats of Sla1p and the EH domain-containing protein End3p (Fig.1.5).

Actin patches could also play a part in cell wall synthesis, as they are concentrated at the sites of cell wall deposition (Adams & Pringle 1984; Kilmartin & Adams 1984) and may also co-localise with cell wall producing enzyme 1,3- β -glucan synthase (Utsugi et al. 2002).

1.3.3 Actin Cables

The second of the actin structures in yeast are actin cables. These align themselves along the mother-bud axis during polarised growth and are involved in the intracellular transport of organelles and vesicles (Adams & Pringle 1984; Drubin, Jones, & Wertman 1993; Schott, Huffaker, & Bretscher 2002), as well as being implicated in the transport of mRNA (Kwon & Schapp 2001). Like actin patches, actin cables also contain a variety of ABPs including:- Sac6p (Fimbrin), Tpm1/2p (Tropomyosin), Bni1p (Formin), and Myo2/4p (class V Myosins) and are thought to assemble at the sites of exocytosis. Transportation along actin cables requires polymerisation, but the Arp2/3 actin nucleation complex is not necessary for this process (Evangelista et al. 2002).

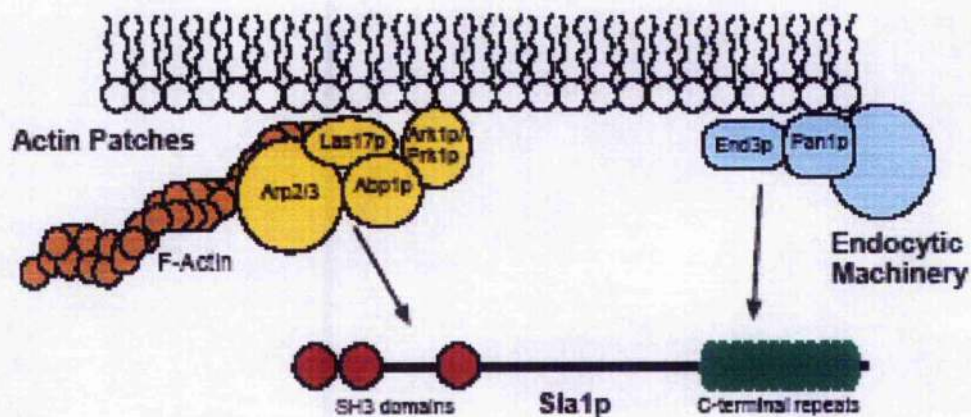


Figure 1.5 A model of actin patch formation

This model reveals how actin dynamics interact with the endocytic machinery. Actin is tethered to the membrane at the cell cortex via Abp1p and Arp2/3 binding to Las17p and Ark1p/Prk1p. Sla1p can then act as a scaffold by binding to both Abp1p and End3p; and integral member of the endocytic machinery. Taken from Warren et al 2002

A model for the formation of these actin cables (Schott, Huffaker, & Bretscher 2002) utilises the Rho-GTPase effector Bni1p which binds at the membrane to formin-binding proteins and also to profilin which recruits actin to the complex allowing the nucleation of an actin filaments to occur (Fig. 1.6). These actin filaments are stabilised by Tpm1/2p and bundled into cables by Sac6p.

1.4 The dynamics of the actin cytoskeleton are controlled by different ABPs

The diverse roles that actin plays in a cell are mediated by the large number of proteins which can bind to either G-actin or F-actin at a variety of binding domains therefore allowing multiple actin binding proteins (ABPs) to bind simultaneously. These ABPs can induce a reorganisation of the cytoskeleton in response to both intra- and extra-cellular signals and can have a variety of different functions (Ayscough 1998; dos Remedios, Chhabra, Kekic, Dedova, Tsubakihara, Berry, & Nosworthy 2003; McGough 1998; Pollard & Borisy 2003) (fig. 1.7). These include:- sequestering G-actin, inducing nucleation of filaments, promoting de-polymerisation, capping filaments, severing F-actin, cross-linking or bundling F-actin filaments, and motor proteins like myosins that can transport cargo along microfilaments. Other ABPs act as scaffolds tethering actin at its site of activity; an example of this is the involvement of Sla1p linking the actin cytoskeleton to the endocytic machinery (Warren, Andrews, Gourlay, & Ayscough 2002).

1.4.1 G-actin binding proteins

One of the most studied of the G-actin binding and sequestering proteins is profilin, a 19 kDa protein (Ampe et al 1988) that is highly expressed in the cytoplasm of eukaryotic cells. It was originally thought to (i) bind and sequester monomeric actin resulting in inhibition of actin filament growth *in vitro* (Carlsson et al. 1977), and (ii) inhibit the hydrolysis of ATP when bound to actin thereby maintaining a pool of actin primed for polymerisation. However it is now known that profilin promotes the exchange of nucleotides in actin monomers from ADP-actin to ATP-actin (Goldschmidt-Clermont et al. 1991) and along with cofilin plays a part in the control of actin filament

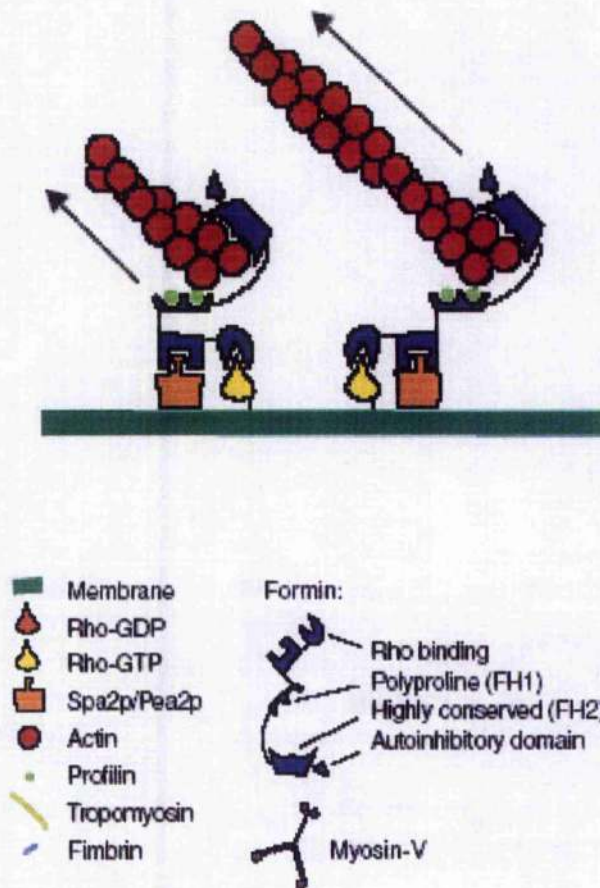


Figure 1. 6 A model of actin cable formation

This model shows a complex arrangement of actin binding proteins. The Rho-GTP binds to formin binding proteins which in turn bind to formin, resulting in a conformational change in formin which allows profilin to bind and recruit actin monomers to the growing actin filament. These filaments are then stabilised by tropomyosin and bundled by fimbrin. Taken from Schott et al 2002.

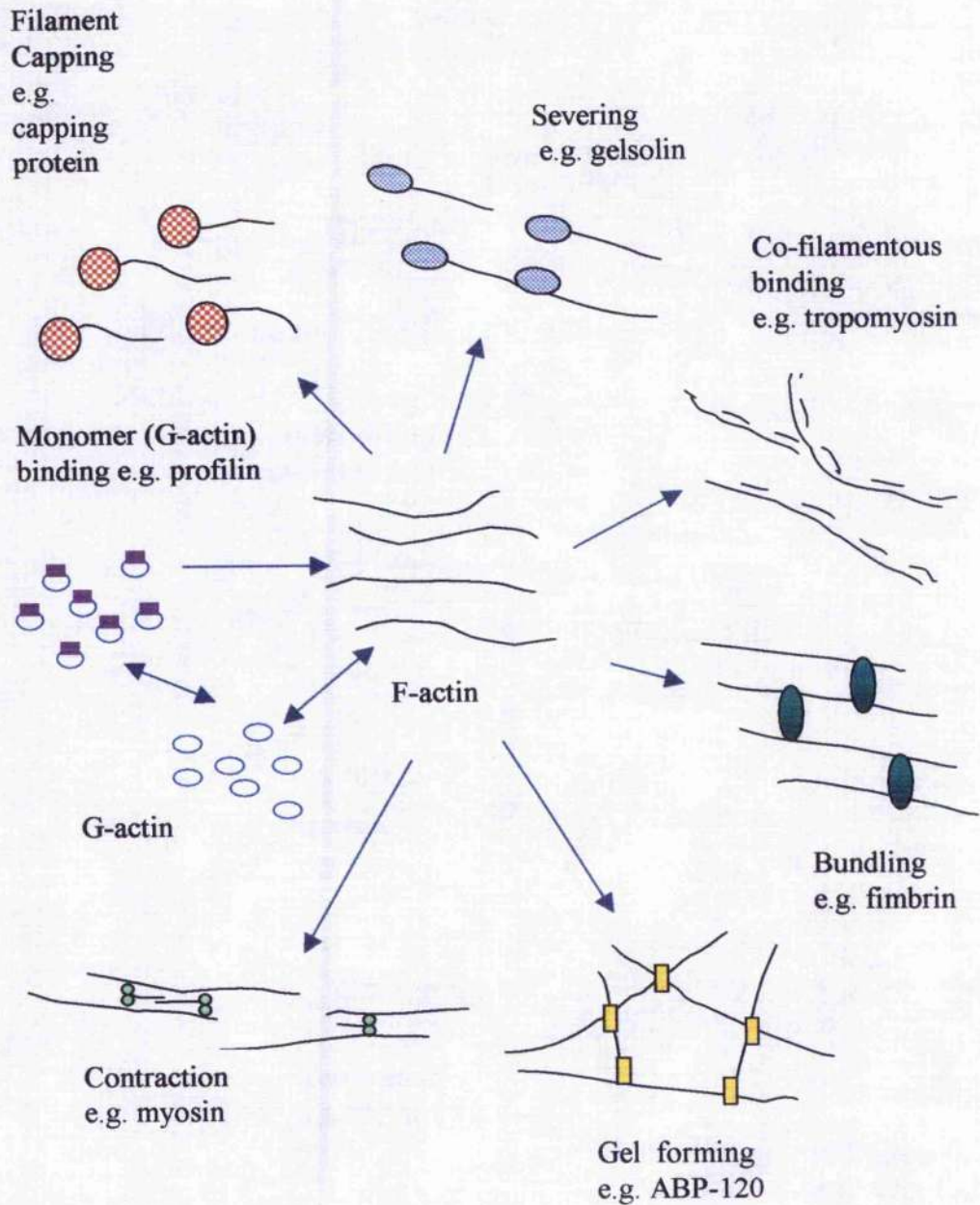


Figure 1.7 The function of actin-binding proteins (ABPs)

ABPs regulates actin filament structure in a variety of different ways. Specific functions of actin-binding proteins as determined by *in vitro* research are shown with a diagram of how each protein may interact with F-actin. Adapted from Ayscough 1998.

treadmilling. Another role for profilin can be seen in the targeting of ATP-actin to sites of filament growth via its ability to bind the formin family of ABPs (Fig. 1.6) (Schott, Huffaker, & Bretscher 2002). It is also worth noting that dissociation of profilin from actin is stimulated by PIP and PIP₂ (Goldschmidt-Clermont et al. 1990), indicating a possible role in the signalling between the cell membrane and the actin cytoskeleton.

1.4.2 Actin depolymerising proteins

Another family of ABPs involved in the control of actin filament treadmilling is the ADF/cofilin family; these 15-19 kDa proteins are expressed throughout eukaryotic cells and exist as multiple isoforms (dos Remedios et al. 2003). Its role in treadmilling is achieved by increasing by about 30 fold the off rate of actin monomers from the pointed end of actin filaments without affecting the off rate at the barbed end (Carrier et al. 1997). The ADF/cofilin complex has also been implicated in the severing of actin filaments (Maciver et al. 1998); this could aid depolymerisation by creating more ends which could disassemble, although this mechanism may require the cooperative binding of two cofilins (Galkin et al. 2001).

The cellular functions of ADF/cofilin appear to be essential for survival since a deletion of the *S. cerevisiae* homologue COF1 proves lethal (Moon & Drubin 1995). ADF/cofilin can be negatively regulated by phosphorylation (Moon & Drubin 1995), and although this is not the case for yeast cofilin, a mutation (Ser³ - Glu) that mimics the inactive state results in lethality (Lappalainen et al. 1997). Other factors important in the regulation of ADF/cofilin are membrane lipids such as PIP and PIP₂, which can be inhibitory, and changes in pH, which can promote depolymerisation in alkaline conditions (Yonezawa & Nishida 1990).

1.4.3 Capping proteins

The control of the length and distribution of filaments relies on the ability of certain ABPs to block the addition and subtraction of actin monomers at either the barbed or the pointed end of the filament. Capping proteins (CAPs) are heterodimeric proteins found in all eukaryotic cells which act to regulate filamentous growth by binding to the barbed

end of filaments and blocking the addition and subtraction of monomeric actin (Schafer, Hug, & Cooper 1995). Capping of the barbed or fast growing end results in a net increase in actin monomers at the pointed end, creating a pool of free G-actin available for the rapid polymerisation necessary for dynamic growth and motility (Hug et al. 1995). Another role for CAP is thought to be in nucleating new filaments; this is achieved by the ability of actin monomers to add to the pointed or slow growing end under the correct conditions (Section 1.2.3). It is also worth noting that CAP can inhibit loss of monomers from the barbed end under depolymerising conditions thereby preventing filament shrinkage. Inhibition of CAP by the phosphoinositides PIP and PIP2 results in its dissociation from the filament creating a pool of free barbed ends available for rapid polymerisation.

In *S. cerevisiae* the heterodimeric capping protein is expressed from 2 genes CAP1 and CAP2 (Amatruda & Cooper 1992). Deletion of these genes is not lethal although they do show synthetic lethality with SAC6 (fimbrin) deletions (Adams, Cooper, & Drubin 1993). An explanation for this is that capping protein and fimbrin act together to control filament stability (Karpova, Tatchell, & Cooper 1995).

1.4.4 F-actin severing proteins

Gelsolin is a protein of molecular weight about 80 kDa, which belongs to a class of ABPs that can bind to F-actin and sever filaments in response to elevated Ca^{2+} levels (Yin, Albrecht, & Fattoum 1981). There are many different members of the gelsolin family; a marker for these proteins is a 120 amino acid repeat sequence of which gelsolin possesses six. Gelsolin has a high affinity for filaments and can cap the barbed ends after severing. It induces a rapid rearrangement of the cytoskeleton by creating a large number of short filaments, which can facilitate polymerisation by increasing the amount of free barbed ends (Cooper & Schafer 2000). Regulation of gelsolin is achieved through PIP2, which induces its dissociation from actin, and intracellular Ca^{2+} levels which not only induce severing but also increase the affinity of gelsolin for PIP2. Over expression studies with gelsolin have also suggested a role in modulating phospholipase C signalling via its ability to compete for PIP2 (Sun, Lin, & Yin 1997).

1.4.5 The Arp 2/3 Complex

The Arp2/3 complex consists of two proteins structurally similar to actin, Arp2p and Arp3p as well as five smaller unrelated (Arc) proteins (Machesky & Gould 1999). It appears to be conserved in all eukaryotes and is associated with regions of dynamic actin assembly such as membrane ruffle (Mullins, Heuser, & Pollard 1998). The main function of the complex is to create branch points by binding to existing filaments and nucleating the assembly of new filaments. This process is called dendritic nucleation and can be stimulated by extra-cellular signals that transmit via G-protein coupled receptors to the WASP (Wiscott-Aldrich syndrome protein) family of proteins (Machesky et al. 1999). Activated WASP binds to the complex of F-actin and Arp2/3 and induces a conformational change, which allows the nucleation of a new filament or the formation of a Y-branch at 70° to the pre-existing one. Due to its structural similarity the Arp2/3 complex can also mimic an actin dimer, which when bound to monomeric actin forms a stable nucleus (Kelleher, Atkinson, & Pollard 1995). This allows for the rapid polymerisation of filaments by by-passing the rate limiting actin dimerisation step (Section 1.2.2). It associates with the Arp2/3 complex

In *S. cerevisiae* the WASP family proteins Las17p/Bee1p, which associate with the Arp2/3 complex are found localised to cortical actin patches. Mutations in these proteins have shown that they are essential for patch formation and motility (Winter et al. 1997), as well as indicating a role for them in both endocytosis (Moreau et al. 1997) (Fig.1.5) and mitochondrial motility (Boldogh et al. 2001).

1.4.6 F-actin Cross-linking or bundling proteins

The actin cytoskeleton can be stabilised by the binding of ABPs and the subsequent cross-linking or bundling of filaments into three-dimensional networks or tight parallel bundles (Ayscough 1998; Otto 1994). In order to achieve this bundling activity an ABP must either contain 2 actin-binding domains (ABD) or exist as a dimer containing 2 ABDs. Fimbrin and α -actinin, both members of the calponin homology (CH) domain superfamily of proteins, have been shown to induce the formation of these higher order actin structures (Otto 1994). The *S. cerevisiae* fimbrin homologue (Sac6p) localises to

both actin patches and cables and has been reported to be involved in regulating actin dynamics, endocytosis, and cell polarity *in vivo* (Adams, Botstein, & Drubin 1991).

1.5 Calponin

Calponin (CaP) is a 34 kDa protein first found in smooth muscle (Takahashi, Hiwada, & Kokubu 1988), and was implicated in the regulation of muscle contractility via its ability to inhibit the actomyosin ATPase activity (Winder & Walsh 1990). To date, three isoforms of this protein have been isolated:- h1 CaP (Takahashi, Hiwada, & Kokubu 1988) found in the contractile apparatus of smooth muscle cells, h2 CaP (Strasser et al. 1993) found in non-muscle cells and thought to be involved in cytoskeletal organisation (Danninger & Gimona 2000), and acidic CaP (Applegate et al 1994) which is found enriched in brain tissues and has been implicated in the regulation of neurite outgrowth (Ferhat et al 2001). Biochemical studies have also shown that smooth muscle CaP accelerates actin polymerisation at low ionic strengths and stabilises actin filaments *in vitro* (Kake et al 1995).

Although the major binding partner of CaP is actin, it also binds to large number of other cytoskeletal proteins including myosin (Szymanski & Tao 1993), tropomyosin (Childs et al 1992), desmin (Fujii et al 200), and α -actinin (Leinweber et al 1999) indicating a structural role within the cell. Calponin can also interact with a variety of second messengers including phospholipids (Fujii et al 1995), Rho-kinase (Kaneko et al 2000), and protein kinase C (Leinweber et al 2000) and has been suggested to play a key role in the linking of signalling pathways to the cytoskeleton

Calponin consists of several recognisable structural domains (Fig. 1.8) including an amino terminal binding site for Ca^{2+} -binding proteins, a calponin homology (CH) domain, three calponin-like repeats (CLR), and a carboxy terminal tail sequence. It also contains two putative actin binding domains (ABDs), one (ABD1) being the region between the CH domain and first CLR and the other (ABD2) spanning the three CLRs, and including the phosphorylation site serine 175 which is thought to be involved in the regulation of actin binding (Winder et al 1993). The three genetic isoforms of calponin are highly homologous with the only major difference being

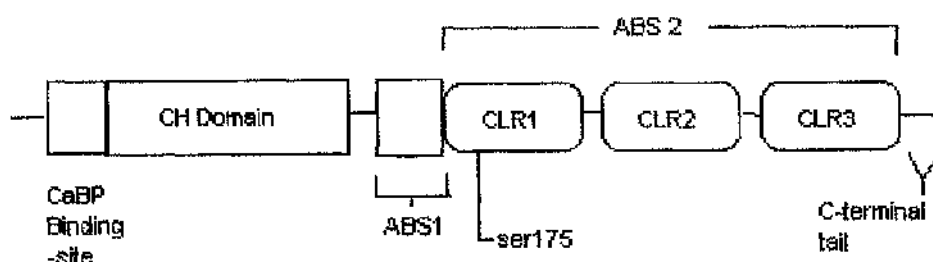


Figure 1. 8 The domain organisation of calponin.

A protein of 297aa with an amino terminal Ca^{2+} /calmodulin binding site; a 95 aa calponin homology (CH) domain; 3 CLIK²³ Calponin Like Repeats; and a carboxy terminal tail sequence. Calponin also has two putative actin binding sites (ABS); ABS1 is located in the region between the CH domain and the first CLR; and ABS2 spans the 3 CLRs incorporating Ser¹⁷⁵ which has been shown to be critical for actin binding. Adapted from Small and Gimona 1998.

seen in the carboxy tail sequence where differences in both the size and charge of the amino acids can be observed. This region is thought to bind to residues in ABD2 and is predicted to have an autoinhibitory effect on actin binding (Danninger & Gimona 2000, Burgstaller et al 2001).

1.5.1 Calponin homology (CH) domains

The CH domain is a protein module of about 100 amino acids which is found in a variety of both cytoskeletal and signalling proteins and was proposed to function as an autonomous actin binding motif (Castresana & Saraste 1995). This hypothesis was based on identification of a 250 amino acid ABD found in cross-linking proteins consisting of two tandem repeats of about 125 amino acids corresponding to two distinct CH domains. The ability of the amino-terminal CH domain of α -actinin to bind actin (Way, Pope, & Weeds 1992) and the use of EM reconstructions to show that the majority of contacts within the ABD of fimbrin are located on the amino-terminal CH domain supported this hypothesis (Hanein, Matsudaira, & DeRosier 1997). Further analysis of these domains resulted in a sub-division into amino-terminal CH1 and carboxy-terminal CH2 domains with CH1 or CH2 domains from different proteins showing greater homology than CH1 and CH2 domains from the same protein.

One problem with this classification of CH domains as prototype actin binding modules is that CH2 domains bind actin either weakly or not at all (Way, Pope, & Weeds 1992; Winder et al. 1995) indicating a redundancy of this CH domain in actin binding. The fact that CH1 of α -actinin binds to actin with a lower affinity than the complete ABD (Way et al. 1992) suggests a stabilising role for the CH2 domain through inter-domain helix-helix interactions and indicates a need for both CH1 and CH2 domains to form a fully functional ABD (Goldsmith et al. 1997b). Further detailed analysis of the CH domain sequence have also shown that it does not overlap the ABD of CaP and that the CH domain in CaP and in its smooth muscle homologue SM22 are not sufficient or necessary for F-actin binding (Gimona & Winder 1998).

A more recent study of CH domains containing proteins (Stradal et al. 1998) classifies them into 3 different groups:- (i) monomeric cross-linking proteins containing two ABD's (fimbrin), (ii) dimeric cross-linking proteins (α -actinin, α -spectrin) and

monomeric actin binding proteins (utrophin, dystrophin) each containing one ABD, and (iii) the largest and most diverse group which contain proteins with only one amino terminal CH domain (CaP, sm22, Vav, IQGAP). The CH domain sequences in these groups of proteins were analysed and classified into a further 5 sub-divisions (Fig. 1.9) with separate branches for CH1 and CH2 domains from 1ABD containing proteins. Likewise CH1 domains from the ABD1 and ABD2 (CH1.1 and CH1.2) of fimbrin are more similar than corresponding CH2 domains (CH2.1 and CH2.2), and both are separated from other CH1 and CH2 domains. Single CH domains (CH3) are also branched separately as they are significantly different from the CH domains that comprise ABDs.

The CH domain structure has been solved from several different proteins including:- spectrin, calponin, fimbrin, and utrophin (Bramham et al. 2002; Carugo, Banuelos, & Saraste 1997; Goldsmith, et al. 1997b; Keep, Norwood, Moores, Winder, & Kendrick-Jones 1999), and reconstructions show a high level of conservation in secondary structure. The CH domain of CaP (Bramham et al. 2002) is made up of four main α -helices (1, 3, 4 and 6) (Fig. 1.10) each comprising between 11 & 18 residues joined by long loops, and two shorter and less regular helices (2 and 5). Helices 3, 4 and 6 form an inner core with the two shorter helices 2 and 5 located together at the N-termini of helices 3 and 6 respectively. Helix 1 is hydrophobically tethered by, and lies perpendicular to, helices 3 and 6, as is helix 5. A 3_{10} -helical turn can also be found in the long loop between helices 4 and 5.

1.5.2 Calponin Like Repeat (CLR)

The calponin like repeats (CLR) or CLIK²³ repeat modules (Lener, Burgstaller, & Gimona 2004) are 23-29 amino acid stretches which have been found on a variety of actin-binding proteins including:- calponin which contains three tandem repeats, the SM22/trangelin family of smooth muscle proteins each of which possess one CLR, and the muscle protein of *C. elegans* UNC-87 which contains seven CLRs (Fu et al. 2000; Gimona & Mital 1998; Kranewitter, Ylanne, & Gimona 2001). These CLRs are

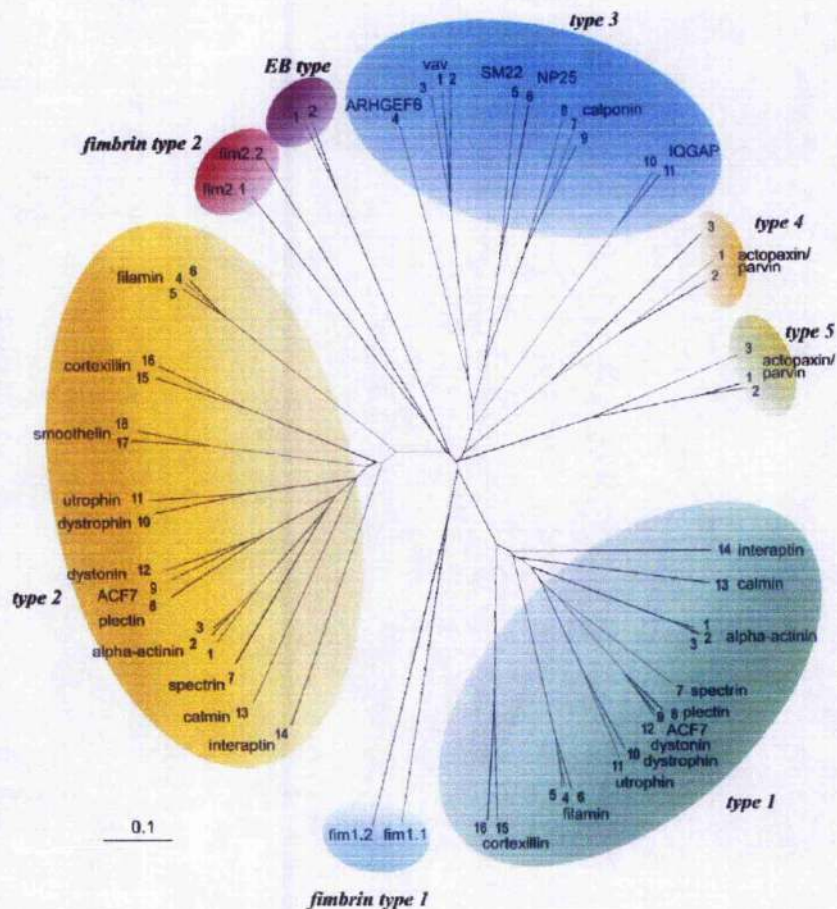


Figure 1. 9 Phylogenetic tree of CH domains.

The analysis includes 104 individual sequences from 60 single and multiple CH domain-containing proteins. CH domains are sub-divided into 5 populations:- Proteins with 2 CH domains (CH1 and CH2) and one ABD; CH1 domains from these proteins share more homology with CH1 domains from other proteins than CH2 domains from the same protein; and *vica versa*. The CH2.1 and CH2.2 domains of fimbrin are also more similar to each other than to the CH1.1 and CH1.2 domains. A separate branch of the tree is occupied with the largest group of proteins; those that possess only 1 CH domain which shares a high degree of homology with other members of the same sub-division. Taken from Gimona et al 2001.

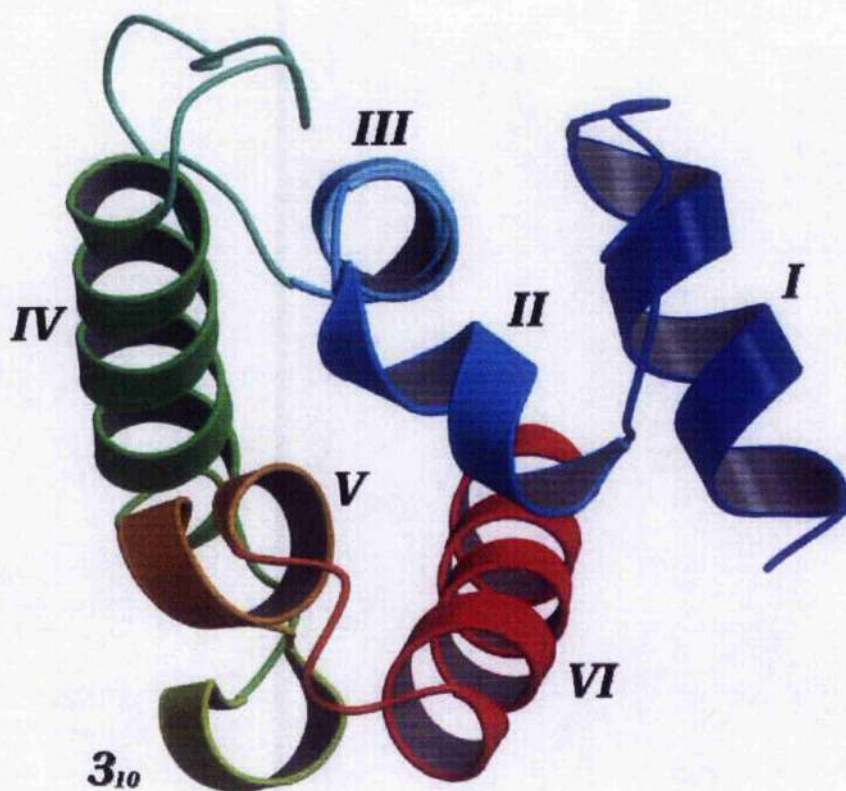


Fig.1.10 Ribbon diagram of CH domain structure

The CH domain of CaP is made up of four main α -helices (1, 3, 4 and 6) each comprising between 11 & 18 residues joined by long loops, and two shorter and less regular helices (2 and 5). Helices 3, 4 and 6 form an inner core with the two shorter helices 2 and 5 located together at the N-termini of helices 3 and 6 respectively. Helix 1 is hydrophobically tethered by, and lies perpendicular to, helices 3 and 6, as is helix 5. A 3_{10} -helical turn can also be found in the long loop between helices 4 and 5. Taken from Bramham et al 2002.

highly conserved at the amino acid level and have been proposed to contain a unique actin-binding interface. In calponin the second ABD spans the three tandem CLRs on the carboxy terminal side of the CH domain (Danninger & Gimona 2000); in SM22 the single CLR which again lies on the carboxy terminal side of a CH domain has been shown to be necessary for actin binding (Fu et al. 2000); and in the case of UNC-87 which does not contain a CH domain the multiple CLRs have been shown to bind actin directly and form tight F-actin bundles (Kranewitter, Ylanne, & Gimona 2001). The CLR module has been identified as the domain responsible for stabilisation of actin filaments by calponin (Burgstaller, Kranewitter, & Gimona 2002); and it has also been shown that the ABD conferred by this module does not compete with other ABD's containing tandem CH domains such as α -actinin (gimona et al 2002).

In the case of calponin, it has also been shown that acidic carboxy terminal tail sequences could play a role in the autoregulation of basic actin-binding to CLRs, which function via an electrostatic interaction to block the ABD2 (Danninger & Gimona 2000). CLRs also contain a conserved serine/threonine residue (CaP Ser¹⁷⁵, SM22 Ser¹⁸¹); which when phosphorylated interferes with CLRs binding to actin (Fu et al. 2000; Winder et al. 1993).

1.6 The smooth muscle protein SM22/transgelin

The smooth muscle protein SM22 was first isolated from chicken gizzard (Lees-Miller et al. 1987) as a protein with a molecular mass of 22 kDa and of unknown function. This was later shown to be the same protein as transgelin (Tgln) a shape-change-sensitive actin-gelling protein found in fibroblasts (Shapland et al 1988). SM22/transgelin has since been identified as WS3-10 in senescent human fibroblasts (Thweatt, Lumpkin, Jr., & Goldstein 1992), and as p27 in mouse (Almendral et al. 1989); and homologues mp20 in *D. melanogaster* (Ayme-Southgate et al. 1989); and UNC-87 in *C. elegans* (Goetinck & Waterston 1994). It is detectable in some primary smooth muscle cultures but is rapidly lost with further time in culture (Solway et al. 1995). SM22/transgelin possesses two distinct domains with significant homology to calponin:- (i) an amino terminal CH domain and, (ii) a carboxy-terminal CLR (figure 1.11). Although SM22/transgelin exhibits significant homology with calponin at the amino acid sequence level, as well as

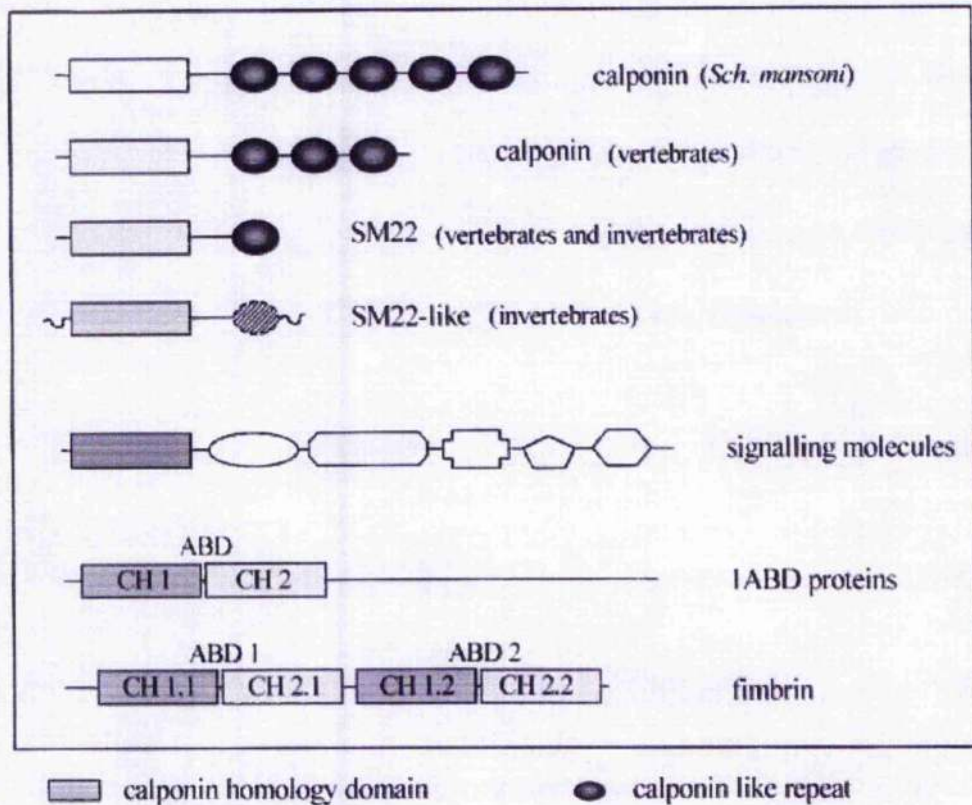


Figure 1. 11 A schematic representation of CH domain containing proteins: The different sub-divisions of the calponin family of proteins and the domains which they share. There is fimbrin which has 4 CH domains and possesses 2 ABDs. Proteins like dystrophin which possess only 1ABD and have 2 CH domains. The largest and most varied sub-division of CH domain proteins is those which possess a single CH domain, these can be further sub-divided with respect to the other domains like CLRs and various signalling motifs that the protein may possess. Taken from Stradal et al 1998.

possessing a similar domain structure, there was some controversy as to whether it could bind actin (Fu et al. 2000) or not (Gimona & Mital 1998).

One possible explanation for this apparent discrepancy between the results could be differences in experimental conditions employed during co-sedimentation assays, with Fu et al. 2000; reporting actin binding at low ionic strength. It has been suggested that there is an actin-binding site on SM22/transgelin, situated between the N-terminal CH and the C-terminal CLR and encompassing residues 154-161 (Prinjha et al. 1994). This putative ABD (154-KKAQEHKR-161) contains four positively charged residues thought to be important in the binding of actin through electrostatic interactions (Fu et al. 2000).

Another possible actin-binding site has been proposed to lie within the CLR and includes the phosphorylation site at Ser¹⁸¹; which *in vitro* has been shown to be phosphorylated by PKC, resulting in an inhibition of actin-binding (Fu et al. 2000). SM22/transgelin also possesses a putative EF hand motif at residues 108-119, which appears to be non-functional as Ca²⁺ has no effect on actin-binding (Lawson, Harrison, & Shapland 1997). SM22/transgelin serves as an early marker of smooth muscle tissue as it appears late in the embryonic development of chicken relative to other components of smooth muscle cytoskeleton. Whereas Sm22/transgelin has been shown to bind actin, the function of this protein in higher eukaryotes has remained uncertain, with mice deficient for the smooth muscle variant SM22 α exhibiting no overt smooth muscle phenotype (Zhang et al. 2001). It is also interesting to note that the expression pattern of this protein in various primary and cultured cells shows it to be a transformation- and age-sensitive protein (Gonos et al. 1998).

1.9 Discovery of a calponin homologue (Scp1p) in *Saccharomyces cerevisiae*

A search of the yeast genome identified a single open reading frame with homology to the calponin family of proteins (Epp & Chant 1997). Sequence analysis of this gene identified a single amino terminal calponin homology (CH) domain, and a single 27 amino acid calponin-like repeat (CLR) (Fig. 1.11) as had been identified previously in SM22/transgelin, a subfamily of CH domain proteins. Indeed Scp1p shares 28% identity and 48% similarity with SM22/transgelin at the amino acid sequence level. The protein Scp1p has a molecular mass of 22 kDa and is basic in nature with a predicted pI of 9.5.

1	PRQELRENIETVTRRIGN	---NPDGLKGGIILGSEINKLP	---SS---VKKINES-TQN	-----HOLENIGNTIKAITK	YGVKPHDIEEANDLFENTHTQV	STELALAS	Vertebrate calponin		
2	TRQRLNIEGTRRRIGG	---NPDGLKGGIILGSEINKLP	---SS---VQKNDP-VQN	-----HOLENIGNTIKAITK	YGVKPHDIEEANDLFENTHTQV	STELALAS			
3	QAEQRLNIEGTRRRIGG	---NPDGLKGGIILGSEINKLP	---SS---VKKINES-TQN	-----HOLENIGNTIKAITK	YGVKPHDIEEANDLFENTHTQV	STELALAS			
4	KAELRTWIEGTRRRIGG	---DFOGLKGGIILGSEINKLP	---SS---VKKINES-TQN	-----HOLENIGNTIKAITK	YGVKPHDIEEANDLFENTHTQV	STELALAS			
5	DLQILQITTTCKDNGR	---PQGRNFM	---LQGGTVLGEINL	---PQAPVKIKGAS-TNA	-----FKQEQISQTLQAAER	YQINT	Vertebrate SM22		
6	ELERAVENIIVCPDNGR	---PQGRNFM	---LQGGTVLGEINL	---PQAPVKIKGAS-TNA	-----FKQEQISQTLQAAER	YQINT			
7	ELERAVENIIVCPDNGR	---PQGRNFM	---LQGGTVLGEINL	---PQAPVKIKGAS-TNA	-----FKQEQISQTLQAAER	YQINT			
8	DLNKLVDWILLCAEDIEH	---PPGRTHQK	---LQGGTVLGEINL	---PQAPVKIKGAS-TNA	-----FKQEQISQTLQAAER	YQINT	Invertebrate SM22		
9	EMKEQAEWIEALIAEKPPA	---QSVEDVLKQGVLCIKLINSIS	---APVKVSSGGQ	-----FKMENINNFQKALKE	YGVDPIDIVFQTVDLTKKQI	AVNTIIFALGR			
10	QNEALENIEALITGLKIDR	---SKLVEDIKQGTVLCKLINSIS	---G---CIKINENA-TWP	-----FKMENINNFQKALKE	YGVDPIDIVFQTVDLTKKQI	AVNTIIFALGR			
11	ESPAETNINQITNENWPF	---RENVAASRMGQIS	---KILNIVFDGTASLP	---AAAKMRPFRANTMTAP	LRUKI	QTTTANAVA	Invertebrate calponin and SM22-like		
12	VEGELNINIKKVTGENTAIN	---GTRNFVKQKQGTLLCKLINSIS	---8---ITRAQAKPNST	-----FOYNNHLELITPIS	---QGVPRASEFQTVDLTKKQI	AVNTIIFALGR			
13	EAQIKINIVKSVLKETAPP	---G---DLECLKQGTVLCKLINSIS	---11---VEADGEANISGKSKMP	-----FVQNDQISQTLQAAER	YQINT	FKQEQISQTLQAAER			
14	EEATEIKNIENVTGERTS	---FQVTTCESSDVS	---NLAQGMVLCIKLINSIS	---8---CRVYKPKPNA	-----FPMENISNTLQAAER	YQINT			
15	VEAGYILENIROLTKEDTCE	---ASRDNFEQKQGTLLCKLINSIS	---8---ITRAQAKPNST	-----FVQNDQISQTLQAAER	YQINT	FKQEQISQTLQAAER			
16	KEATLLENIKILSGENIST	---GERDNFNILKQGTLLCKLINSIS	---8---ITRAQAKPNST	-----FVQNDQISQTLQAAER	YQINT	FKQEQISQTLQAAER			
17	NLAGETIQWQVTTQSDTQ	---GDADNIVKQGTLLCKLINSIS	---8---ITRAQAKPNST	-----FVQNDQISQTLQAAER	YQINT	FKQEQISQTLQAAER			
18	QLEKARENIEETLTKENAO	---LDLLDQKQSVILCKLINSIS	---8---ITRAQAKPNST	-----FVQNDQISQTLQAAER	YQINT	FKQEQISQTLQAAER			
19	---EQIVTWLISGLVSPKTKICDP	---EEFLKSSKNGVILCKLINSIS	---8---ITRAQAKPNST	-----FVQNDQISQTLQAAER	YQINT	FKQEQISQTLQAAER	VAV and VAV-like		
20	ELNRQCTHLLIQCVLPSPHRTVMDGQVCELAQALRGVLLQGLNLLPHAI	---NLAQGMVLCIKLINSIS	---8---ITRAQAKPNST	-----FVQNDQISQTLQAAER	YQINT	FKQEQISQTLQAAER			
21	ELNRQCTHLLIQCVLPSPHRTVMDGQVCELAQALRGVLLQGLNLLPHAI	---NLAQGMVLCIKLINSIS	---8---ITRAQAKPNST	-----FVQNDQISQTLQAAER	YQINT	FKQEQISQTLQAAER			
22	DLMTGCARLRDMKVLTTDK	---NGTMLFASVLRGGLLQGLNLLPHAI	---NLAQGMVLCIKLINSIS	---8---ITRAQAKPNST	-----FVQNDQISQTLQAAER	YQINT	IQGAP and IQGAP-like		
23	CHLEAKRWNEACLEGEDP	---PTTELEEGLRNGVYLAQALRGVLLQGLNLLPHAI	---NLAQGMVLCIKLINSIS	---8---ITRAQAKPNST	-----FVQNDQISQTLQAAER	YQINT			
24	CHLEAKRWNEACLEGEDP	---PTTELEEGLRNGVYLAQALRGVLLQGLNLLPHAI	---NLAQGMVLCIKLINSIS	---8---ITRAQAKPNST	-----FVQNDQISQTLQAAER	YQINT			
25	CRVDEAKNIEELATDLG	---PTSTEQSLRGVYLAQALRGVLLQGLNLLPHAI	---NLAQGMVLCIKLINSIS	---8---ITRAQAKPNST	-----FVQNDQISQTLQAAER	YQINT			
26	CRVDEAKNIEELATDLG	---PTSTEQSLRGVYLAQALRGVLLQGLNLLPHAI	---NLAQGMVLCIKLINSIS	---8---ITRAQAKPNST	-----FVQNDQISQTLQAAER	YQINT			
27	CRVSEVKINIEAVIEEALPSEIE	---PTTDLLENIRNGVYLAQALRGVLLQGLNLLPHAI	---NLAQGMVLCIKLINSIS	---8---ITRAQAKPNST	-----FVQNDQISQTLQAAER	YQINT			
1	h1 calponin, human	6	SM22, human	16	1938530, <i>C. elegans</i>	21	VAV2, human	26	F09C3.1, <i>C. elegans</i>
2	h1 calponin, chicken	7	SM22, chicken	17	F4309.9, <i>C. elegans</i>	22	746334, <i>C. elegans</i>	27	YPL242c, <i>S. cerevisiae</i>
3	acidic calponin, human	8	NP25, rat	18	2130827, <i>C. elegans</i>	23	IQGAP 1, human		
4	h2 calponin, human	9	mp20, <i>D. melanogaster</i>	19	435446, human	24	IQGAP 2, human		
5	KIAA0120, human	10	myophilin, <i>E. granulosus</i>	20	VAV, human	25	2130829, <i>Schiz. pombe</i>		

Figure 1. 12 Sequence alignment of CH domains of proteins which contain a single CH domains: reveals a high degree of homology. The residues marked in grey are conserved in >50 % of the proteins. Residues conserved in sub-families are as follows:- yellow, IQGAP; blue, VAV; green, vertebrate CaP; pink, vertebrate SM22. Conserved residues between vertebrate CaP and SM22 and, vertebrate and invertebrate SM22 are shown in red. Taken from Stradal et al 1998.

1.10 Aims of the present work

The functions of the SM22/transgelin family of proteins within the cell have yet to be fully understood, with conflicting reports surrounding their role in actin-binding/bundling (Fu et al. 2000; Gimona & Mital 1998) and SM22 knockout mice showing no discernible smooth muscle phenotype (Zhang et al. 2001). In this work, the aims were to:- (i) purify Scp1p, a member of the SM22/transgelin family; (ii) use a range biochemical techniques to identify and characterise actin-binding properties of Scp1p; and (iii) identify a role for Scp1p *in vivo*.

2. Materials and Methods

2.1 Molecular Biology Techniques

2.1.1 Restriction enzyme digestion of DNA

All enzymes used in this study were from New England Biolabs and digests were carried out according to the manufacturer's instructions, using the buffers supplied. Digests were typically performed in a final volume of 20 μ l, using 2-10 units of enzyme per reaction. Double digests were performed where necessary when buffer conditions were compatible keeping the volume of enzyme added per reaction under 10% of the reaction volume. Nuclease-free water was added as required to make up the appropriate final volume. Most digests were carried out at 37°C for 2-3 hours unless otherwise recommended by the manufacturer.

2.1.2 Plasmid DNA purification using the Qiagen preparation system

Purification of plasmid DNA was prepared using the Qiagen DNA purification system (mini, midi, maxi or giga) according to the manufacturer's instructions. This system was based on the alkaline lysis method of DNA purification followed by purification of the DNA on a silica-based resin. The DNA was eluted from the resin in 50 μ l of 1 X TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) or sterile distilled water. All plasmid DNA was purified in this way if it was needed for DNA sequencing or for use in transformations.

2.1.3 Electrophoresis of DNA using agarose gels.

DNA fragments were routinely separated and visualised using flat bed agarose gel electrophoresis. Agarose gels were made by melting 0.8 % agarose in TAE (10 mM Tris/HCl, 1 mM EDTA, pH 7.5). 5 μ l of a 10 mg ml⁻¹ stock of ethidium bromide was added per 50 ml of agarose solution. The solution was then poured into a casting tray

containing a comb with the required number of teeth. After the gel had set, the DNA was mixed with 6x gel loading solution (0.25 % Bromophenol Blue, 0.25 % Xylene Cyanol FF, 30 % glycerol in water) and then loaded into the wells of the gel. Samples were subjected to electrophoresis in 1 x TAE at between 80 and 120 V until the bromophenol blue of the loading solution had run between 2.5 and 5 cm. The DNA was then visualised by illuminating the gel with an UV light source.

2.1.4 DNA ligation

DNA ligation was performed using a Rapid DNA ligation kit (Roche Diagnostics). Vector and insert DNA were diluted in DNA dilution buffer to a final volume of 10 μ l. 10 μ l of 2 X DNA ligase buffer was then added along with 1 μ l of T4 DNA ligase. A control reaction containing cut vector and no insert DNA was also set up. The contents were mixed well and incubated at room temperature for 5 min. 2 μ l of the ligation mix was used to transform ultra-competent XL2 blue *E. coli* cells (Stratagene).

2.1.5 Amplification of DNA using the polymerase chain reaction

The polymerase chain reaction (PCR) is a method used to amplify specific DNA fragments using oligonucleotide primers complementary in sequence to the regions flanking the sequence of interest. PCR was used on several occasions during this project; the method described here was a general PCR method used to amplify sequences from plasmids designed to allow deletion or tagging of genes within the yeast genome. Routinely PCR carried out during this study was performed using Bioline reagents. For each PCR reaction the following were mixed in 0.5 ml thin-walled PCR tubes: 1 X *Taq* reaction buffer, 200 nM dNTP's, 1.5 mM $MgCl_2$, 1 μ M forward primer, 1 μ M reverse primer, 1 μ l template DNA, 1 μ l Bioline *Taq* (5 U μ l⁻¹) and water to 100 μ l. The components were mixed gently. A general PCR occurred over 30 cycles of amplification in a MWG Biotech Primus PCR machine, each involving steps of 94°C (1 min), 55°C (2 min), 72°C (1-4 min). Cycling parameters varied according to length of final product: 1-minute extension time (72 °C) was allowed per 1 kb to be amplified. A final elongation step was used at the end of the amplification cycles by incubating at 72°C for 5 min. The

resulting product was analysed by electrophoresis on a 0.8 % agarose gel to detect the amplification products.

2.1.6 Bacterial growth media

The following media were used for the growth and maintenance of various *E. coli* strains used throughout this study.

2xYT (1.6 % tryptone, 1 % yeast extract, 0.5 % NaCl in water) for liquid medium and 2xYT with 2 % agar added for solid medium.

When preparing media for growth of *E. coli* strains harbouring a plasmid conferring antibiotic resistance, the medium was supplemented with the appropriate antibiotic. Ampicillin (100 mg ml⁻¹ stock) was added to a final concentration of 100 µg ml⁻¹.

2.1.7 Preparation of calcium-competent DH5α cells.

An overnight culture of DH5α cells was diluted into 100 ml of 2xYT liquid. Cultures were then incubated at 37 °C until the A₆₀₀ reached 0.5-0.6. The culture was split into 50 ml centrifuge tubes and placed on ice for 10 min. Cells were harvested by centrifugation in a Heraeus megafuge at 600 g for 5 min at 4 °C. The cell pellet was re-suspended in 50 ml of ice cold 100 mM CaCl₂ and incubated on ice for between 30 min and 4 h. Cells were again harvested by centrifugation and the cell pellet was resuspended in 5 ml CaCl₂ + 15 % glycerol. 50 µl aliquots were snap frozen in liquid nitrogen and stored at -80 °C.

2.1.8 Transformation of DNA into *E. coli*

1 µl of DNA was added to 50 µl of competent *E. coli* cells (either DH5α or BL-21); this was incubated at 4°C for 1 hr, before being heat shocked at 42°C for 2 min. Cells were allowed to recover by adding 500 µl of 2xYT media and incubating at 37°C for 1 h and then centrifuged at 6000 g for 2 min, pellets were re-suspended in 100 µl of 2xYT media

and plated out on 2xYT plates containing appropriate antibiotics. The plates were then incubated at 37°C overnight.

2.1.9 Bacterial glycerol stocks glycerol stocks

For long-term storage of bacteria, 1 ml culture was mixed with 1 ml 50 % sterile glycerol in a cryovial and then stored in a -80°C freezer

2.2 Protein Purification Techniques

2.2.1 Expression of protein from *E. coli*

E. coli cells transformed with an expression plasmid containing the protein of interest were grown at 37°C to an A_{600} of 0.8, and then induced by the addition of IPTG to a final concentration of 0.5 mM for between 4 and 16 h depending on levels of expression achieved. 1 ml samples were taken before and after induction to check for expression of desired protein.

2.2.2 Purification of expressed proteins from *E. coli* inclusion bodies

1 litre cultures of *E. coli* induced to express appropriate proteins were centrifuged at 4000g for 20 min, the supernatant was discarded and the pellet was frozen at -20°C. This pellet was then suspended in 50 ml of SET solution (25% Sucrose, 20 mM Tris/HCl pH 8.0, 1 mM EDTA) containing 5 mg lysosyme and a protease inhibitor cocktail, and stirred at room temperature for 30 min. 50 µl of MnCl₂ (1 M), 0.5ml MgCl₂ (1 M), and 5 µl of DNase was added to the suspension, which was then stirred for a further 10 min at room temperature. The suspension was centrifuged at 20000 g for 20 min and the supernatant discarded. The pellet was resuspended in 50 ml TDEN solution (20 mM Tris/HCl pH 8.0, 2 mM EGTA, 0.2 M NaCl, 1 % deoxycholic acid) and centrifuged at 8000 g for 5 min. The pellet was resuspended in 50 ml TDE solution (20 mM Tris/HCl

pH 8.0, 1 mM EGTA, 0.25 % deoxycholic acid) and centrifuged at 8000 g for 5 min. The washing with TDE solution and centrifugation step was repeated 5-7 times, or until the pellet was no longer white but grey. At this point the pellet was dissolved in 20 ml 8 M urea, 20 mM Tris/HCl pH 8.0, 1 mM EGTA, and then diluted to a final concentration of 6 M with 20 mM Tris/HCl pH 8.0, 1 mM EGTA. This solution was centrifuged at 50000 g for 1 h at 4°C, and the supernatant consisting of soluble inclusion body proteins was retained.

2.2.3 Ion exchange chromatography

An ion exchange column was prepared using CM-CL6B Sepharose resin which had been washed with a buffer appropriate for the particular protein being purified (6 M urea, 20 mM Tris/HCl pH 8.0, 1 mM EGTA for Scp1p purification). The column was equilibrated with the 3 volumes of the same wash buffer, and then the protein sample was pumped onto the column at a rate of 50 ml/min. The column was then washed with this buffer until a peak representing the unbound protein eluted. At this point a 1-500 mM NaCl gradient dissolved in the same buffer was applied to the column and the eluted protein collected in 5 ml fractions and analysed using SDS-PAGE.

2.2.4 Gel filtration chromatography

A gel filtration column was poured using Sephacryl S-200 resin, which had been washed with a buffer appropriate for the particular protein being purified (6 M urea, 20 mM Tris/HCl pH 8.0, 1 mM EGTA for Scp1p purification). The column was then equilibrated with 2 volumes of the same wash buffer. Samples of proteins to be purified were concentrated using a Micro-con concentrator to approximately 5 ml before being loaded onto the column; the column was then washed with the same buffer until the protein had been completely eluted. Fractions (5 ml) were collected from when the sample had been loaded and analysed by SDS-PAGE.

2.2.5 Hydroxyapatite column purification

Hydroxyapatite resin was washed in a buffer appropriate for the protein being purified (6 M urea, 20 mM Tris/HCl pH 8.0, 1 mM EGTA for Scp1p purification). It was allowed to settle, and the fine particles poured off, before the resin was poured into a column. This was then equilibrated with 2 volumes of the same wash buffer. Before loading samples were concentrated down using a Micro-con concentrator to approximately 5 ml; after this the column was washed in 1 volume of buffer before the protein was eluted using a 1-100mM K_2HPO_4 gradient dissolved in the same buffer. The column flow rate was kept at or below 20 ml/h to prevent compression of the resin. Fractions (5 ml) were collected and then analysed then analysed by SDS-PAGE.

2.2.6 Production of anti-Scp1p antibody

250 μ l of purified Scp1p (0.75 mg ml^{-1}) was mixed in equal volumes with 2 x sample buffer (detailed in section 2.5.1), and loaded into a well of a 12.5 % protein gel (Biorad protean II system). This was then run for 16 h at 9 V in 1 x running buffer (detailed in section 2.5.2) before being stained with Ponceau red to visualise the purified protein band. The band was cut out and finely ground up after freezing in Liquid Nitrogen. This material was split into 4 aliquots and sent to the Scottish Antibody Production Unit (SAPU), Carlisle, Lanarkshire for injection into rabbits.

2.2.7 Purification of anti-Scp1p antibodies, (a) Pre-coupling to beads

1g CNBr-Sepharose-4B was taken and swollen and washed with 200 ml 1 M HCl for 15 min. The beads were then washed with 200 ml Scp1p-buffer (10 mM Mes pH 6.0, 1 mM EGTA), and incubated with 7 ml purified Scp1p (0.288 mg ml^{-1}) for 2 h at room temperature. The mixture was centrifuged at 4000 g and the supernatant removed. The A_{280} was checked to make sure that at least some protein had bound to the beads. The beads were then washed twice in Scp1p-buffer (7 ml), and then blocked by reaction with 0.1 M Tris/HCl pH 8.0 for 16 h at 4°C. The blocked beads were then washed with 100

ml 0.1 M NaOAc, 0.5 M NaCl pH 4.0 and then with 100 ml 0.1 M Tris/HCl, 0.5 M NaCl, pH 8.0. The last 2 washes were repeated twice more before the beads were stored at 4°C in 3.5 ml 0.1 M Tris/HCl, 0.5 M NaCl pH 8.0.

2.2.8 Purification of anti-Scp1p antibodies, (b) Preparation of column

The pre-coupled Scp1p-CNBr Sepharose (approx. 2.5 ml) was poured into a 10 ml column and washed with 25 ml of each of the following buffers (i) 10 mM Tris/HCl, 0.5 M NaCl pH 7.5, (ii) 1 M glycine pH 2.5, (iii) 10 mM Tris/HCl pH 8.8, (iv) 0.1 M Triethylamine pH 11.3, (v) 10 mM Tris/HCl pH 7.5. The washed column was stored at 4°C.

2.2.9 Purification of Scp1p anti-antibodies, (c) Affinity purification

Serum was diluted to 10 ml with 10 mM Tris/HCl pH 7.5 and this was passed through the affinity column 3 times and the column was then washed with 30 ml of (i) 10 mM Tris/HCl pH 7.5, (ii) 10 mM Tris HCl/HCl, 0.5 M NaCl pH 7.5. The bound acidic proteins were eluted with 10 ml of 0.1 M glycine/HCl pH 2.5; 1 ml fractions were collected into tubes containing 0.1 ml 0.1 M Tris/HCl pH 8.0. The column was then washed with 30 ml 10 mM Tris/HCl pH 8.8, before the bound basic proteins were eluted with 5 ml 0.1M Triethylamine pH 11.3. Fractions (1 ml) were collected into tubes containing 0.1 ml 0.1M Tris pH 8.0. The A_{280} of each fraction was measured and those fractions which contain protein (antibody) were pooled, before being dialysed overnight against PBS (50 mM Na_2HPO_4 , 150 mM NaCl pH 7.5).

2.2.10 Purification of GST-fusion and 6x HIS tagged proteins

These were grown up as 1 litre cultures and purified according to the manufacturers recommendations, GST-fusion proteins (pGEX -Amersham Bioscience), 6xHis-tagged (pET – Novagen).

92.3 Actin-binding experiments

2.3.1 Falling Ball Assay

Actin (5-10 μM) was mixed with Scp1p ranging in concentration from 0-10 μM , in a total volume of at least 90 μl (made up with Scp1p-buffer) and polymerisation was initiated with the addition of 10 x KME mix (1 M KCl, 20 mM MgCl₂, and 10 mM EGTA). Some of this mixture was immediately drawn up into a 100 mm x 1 mm diameter capillary tube and allowed to polymerise for at least 2 h; the remaining reaction was set aside for sedimentation assay (Sections 2.3.2 and 2.3.3). After polymerisation had taken place a 1 mm diameter ball bearing was placed at the top of the capillary tube, and the time it took to travel the 100 mm down the capillary was recorded.

2.3.2 Low speed sedimentation assays

Actin (5-10 μM) was mixed with Scp1p ranging in concentration from 0-10 μM , in a total volume of at least 45 μl (made up with Scp1p-buffer) and polymerisation was initiated with the addition of 10 x KME mix. The mixture was then allowed to polymerise for at least 2 h, before being centrifuged at 13000 g for 15 min. The supernatant was transferred to a fresh tube, and both it and the pellet fraction were made up to the same volume with sample buffer before being analysed by SDS-PAGE and stained with Coomassie Blue in order to quantitate the amount of protein in each of the fractions.

2.3.3 High speed sedimentation assays

Actin (5-10 μM) was mixed with Scp1p ranging in concentration from 0-10 μM , in a total volume of at least 90 μl (made up with Scp1p-buffer) and polymerisation was initiated with the addition of 10 x KME mix. The mixture was then allowed to polymerise for at least 2 h, before being centrifuged at 100000 g for 15 min. The supernatant was transferred to a fresh tube, and both it and the pellet fraction were made up to the same

volume with sample buffer before being analysed by SDS-PAGE and stained with Coomassie Blue in order to quantitate the amount of protein in each of the fractions.

2.3.4 Electron microscopy

Actin was mixed with Scp1p (1 μM) or Scp1p buffer in a volume of 50 μl (made up with Scp1p buffer) and polymerisation initiated by addition of 10 x KME. The mixture was then allowed to polymerise for at least 2 h, before being spotted onto a carbon formvar-coated disc and the excess fluid absorbed off using a piece of tissue. This was then stained with 5 μl Nanovan (Universal Biologicals); again the excess fluid was absorbed with a piece of tissue. The contents of the disc were then visualised on a Zeiss 902 electron microscope.

2.3.5 Pyrene actin polymerisation/depolymerisation assay

Actin (1 μM) was mixed with pyrene-labelled actin (supplied by Dr S.J. Winder). Scp1p was then added at concentrations ranging from 0-5 μM and polymerisation was initiated with addition of 10 x KME mix. The resulting rates of polymerisation were measured using a Perkin Elmer LS-50B spectrofluorimeter over the course of 1000 s with the parameters set at: excitation wavelength = 366 nm, emission wavelength = 387 nm and slit widths = 4.0 nm. Measurements were also taken after 24 h to analyse steady state kinetics. Depolymerisation was achieved by a 1:10 dilution of the polymerised actin solution into a buffer lacking polymerisation mix.

2.4 Fluorescence microscopy techniques

2.4.1 Rd-phalloidin staining of actin

Yeast strains were grown up in YPAD liquid medium (detailed in section 2.6.1) to an A_{600} 0.1-0.3 and a 1 ml sample was placed in a fresh Eppendorf tube. 0.134 ml 37 %

formaldehyde was added and the mixture was left at room temperature for 1 h. The cells were centrifuged at 3000 *g* for 5 min and washed twice in 1 ml PBS, 1 mg ml⁻¹ BSA, 0.1 % TritonX-100 with the final re-suspension in 50 µl PBS, 1 mg ml⁻¹ BSA, 0.1 % TritonX-100 to which 5 µl of Rd- phalloidin (40 mg ml⁻¹) was added. This was then left to incubate for 30 min in the dark. After this the cells were washed twice with 1 ml PBS containing 1 mg ml⁻¹ BSA and then re-suspended in 200 µl of this buffer. 20 µl of this suspension was left to settle on the well of a slide pre-coated with poly-L-lysine for 10 min before being washed 3 times on the slide with PBS containing 1 mg ml⁻¹ BSA. Mounting solution (1 drop) was added to the slide, a glass coverslip placed on top, and the sides then sealed with nail varnish. Cells were examined by fluorescence microscopy either immediately or after storage at -20°C until convenient.

2.4.2 Immunofluorescence

Yeast strains were grown up in YPAD liquid medium (detailed in Section 2.6.1) to an A₆₀₀ 0.1-0.3 and a 5 ml sample was placed in a fresh Falcon tube. 0.67 ml 37 % formaldehyde was added and the mixture left at room temperature for 1 h. The cells were centrifuged at 3000 *g* for 5 min and washed twice with 2ml sorbitol buffer (1.2M sorbitol, 0.1M potassium phosphate buffer, pH 7.5) before being re-suspended in 0.5ml sorbitol buffer to which 20µl 1 mg ml⁻¹ zymolyase and 1 µl of 2-mercaptoethanol was added. The suspension was left to incubate for 30-40 min at 37°C. 20 µl of this suspension was left to settle on the well of a slide pre-coated with poly-L-lysine for 10 min before was removed by aspiration. The cells were then permeabilised with 10 µl 0.1 % SDS for 30 s before being washed 10 times per well with PBS containing 1 mg ml⁻¹ BSA. Care was taken not to let the wells on the slide dry out during this procedure. The primary antibody was diluted as necessary in PBS containing 1 mg ml⁻¹ BSA and 15 µl placed on each well and left to incubate for 1 h. Each well was then washed 10 times with PBS containing 1 mg ml⁻¹ BSA before 15 µl of the secondary antibody (diluted as necessary in PBS containing 1 mg ml⁻¹ BSA) was added. The slide was incubated for 1 h in the dark, so as to not bleach the fluorophore, and the wells washed a further 10 times with PBS containing 1 mg ml⁻¹ BSA. Mounting solution (1 drop) was added to the slide, a glass cover slip placed on top and the sides then sealed with nail varnish. Cells were examined by fluorescence microscopy either immediately or after storage at -20°C until convenient.

2.4.3 Mounting medium containing DAPI for staining of DNA

100 mg *p*-phenylenediamine was dissolved in 10 ml PBS (pH 9.0). 90 ml glycerol was added to this solution and the mixture was stirred until homogeneous. 2.25 μ l DAPI stock (1 mg ml⁻¹ 4',6'-diamidino-2-phenylindole dihydrochloride in water) was added and mounting medium was stored in the dark at either -80°C (long term) or -20°C (short term).

2.4.4 Adhering cells to microscope slides

To prevent cells from moving during photography they were fixed to the slide surface with poly-L-lysine as follows: 10 μ l 1 mg ml⁻¹ poly-L-lysine was placed on each well of a slide and allowed to incubate for 1 to 2 min before being rinsed off with distilled water and air dried.

2.4.5 Viewing cells by fluorescence microscopy

Cells were viewed with an Olympus BX-60 fluorescence microscope with a 100 W mercury lamp and an Olympus 100x Plan-NeoFluar oil-immersion objective. Images were captured using a Roper Scientific MicroMax 1401E cooled CCD camera using Scanalytics IP lab software on an Apple Macintosh 7300 computer.

2.5 Protein methods

2.5.1 Preparation of 2 x Sample Buffer

This contains:-

2.5 ml 0.5 M Tris/HCl pH 6.8	2.0 ml H ₂ O
2.0 ml 10 % (w/v) SDS	1.0 ml 2-mercaptoethanol
2.0 ml glycerol	0.5 ml 0.5 % (w/v) bromophenol blue solution

2.5.2 SDS-PAGE

The appropriate percentage of acrylamide gel was poured according to the recipes below.

Separating gel:

	7.5 %	10 %	12 %
Sterile water	4.10 ml	3.44 ml	2.69 ml
30 % (w/v) acrylamide 0.8 % (w/v) bisacrylamide	2.25 ml	3.00 ml	3.75 ml
1.5 M Tris-HCl (pH 8.8), 0.4 % (w/v) SDS	2.25 ml	2.25 ml	2.25 ml
10 % ammonium persulfate in H ₂ O	31 µl	31 µl	31 µl
TEMED	5 µl	5 µl	5 µl

5 % Stacking gel:

Sterile water	1.71 ml
30 % acrylamide, 0.8 % bisacrylamide	0.50 ml
0.5 M Tris/HCl (pH 6.8), 0.4 % (w/v) SDS	0.75 ml
10 % ammonium persulfate in H ₂ O	35 µl
TEMED	3.5 µl

Gels were run in 1 x running buffer (0.025 M Tris/HCl, 0.192 M Glycine, 0.1 % (w/v) SDS, pH 8.6) at 120 volts for approximately 1.5 hours, until the dye front was close to the bottom of the gel.

2.5.3 Coomassie blue staining of SDS-polyacrylamide gels

After electrophoresis, the gel was soaked in Coomassie stain solution (0.2 % (w/v) Coomassie Brilliant Blue R250, 50 % (v/v) methanol, 7 % (v/v) acetic acid, 43 % (v/v) H₂O) for 5 min with continuous movement on a rocking platform. The gel was then de-stained using several changes of 50 % (v/v) methanol, 7 % (v/v) acetic acid, 43 % (v/v) H₂O until the protein bands became clearly visible.

2.5.4 Western blotting

Proteins were separated by SDS-PAGE as described (sections 2.5.2 and 2.5.3). PVDF (Schleicher and Schuell) was soaked in methanol while the filter paper and two pieces of Whatman 3MM paper cut to the same size as the gel were soaked in ice-cold blotting buffer (10 mM CAPS, 10 % (v/v) methanol, pH 11). The blot sandwich was assembled (Whatman 3MM paper, PVDF, gel, Whatman paper) keeping the assembly wet and taking care to exclude air bubbles. The sandwich was then placed in the blotting apparatus, with the membrane against the anode. An ice pack was placed in the apparatus to keep the blotting buffer cool and transfer was performed at 400 mA for one hour.

2.5.5 Western blot detection; Enhanced Chemi-Luminescence (ECL)

Proteins were blotted as described (section 2.5.4). The wet membrane was blocked at room temperature for about 1 h, or overnight at 4°C, in 5 % (w/v) milk powder in TBST (50mM Tris/HCl, 150 mM NaCl, 0.2 % (v/v) Tween-20 pH 7.4). The membrane was then rinse briefly in 1 x TBST then sealed in flat plastic tubing (cut to about the same size as the membrane) with 2 ml blocking solution plus the primary antibody. The membrane was incubated in the presence of primary antibody 1 h at room temperature. The blot was

then removed from the tubing and washed with TBST (100 ml) briefly followed by three 10 minute washes in TBST (100 ml). The membrane was sealed in flat plastic tubing (cut to about the same size as the membrane) with 2 ml blocking solution containing secondary antibody conjugated to horseradish peroxidase for between 30 min and 1 h at room temperature. The membrane was removed from the plastic tubing then washed 3 times with TBST (100ml) for 15 min each time.

Equal volumes (5 ml) of developing reagents (Solution I: 250 mM luminol (3-aminophthalhydrazide (Fluka) in DMSO, 90 mM p-coumaric acid in DMSO, 0.1 M Tris HCl pH 8.5; Solution II: hydrogen peroxide, 0.1 M Tris/HCl pH 8.5) were mixed in the dark room and the membrane was incubated in the developing solution for at least 1 min. The reagents were then drained off and the membrane sealed between sheets of clear plastic film. The membrane was exposed to X-ray film (Kodak) in a cassette and the film was developed in a Xomat automatic developer.

2.6 Yeast Methods

2.6.1 Yeast Growth Media

The following media were used for the growth and maintenance of *S. cerevisiae* strains used throughout this study.

Drop out media

Synthetic minimal medium is supplemented with the appropriate amino acids for complementation of any auxotrophic deficiencies. When selecting for a plasmid or gene marked with an auxotrophic marker, the relevant supplement was omitted from the medium (i.e. dropped out). Synthetic complete mixture (containing all amino acids except those required for the auxotrophic selection of plasmids) was prepared and added to the medium.

0.67% nitrogen base without amino acids (Difco)

Drop out mix (added as directed)

Solid media were supplemented with 2% (w/v) agar.

YPA

1% (w/v) yeast extract

2% (w/v) peptone

0.02% (w/v) adenine

Solid media were supplemented with 2% (w/v) agar.

Sporulation media

1% (w/v) potassium acetate

2% (w/v) agar

Carbon source

YPA and drop out media were routinely supplemented after sterilisation with glucose (sterilised 40% (v/v) stock solution) to 2% (v/v) final concentration and the letter D (dextrose) added to the end of the media it supplements.

Antibiotic supplement

Where cells were transformed with DNA conferring G418 resistance (KanMx marker), G418 (200 mg ml⁻¹ stock solution) was added to a final concentration of 200 µg ml⁻¹. Media were cooled to 50°C prior to addition of antibiotic. Poured plates were immediately stored at 4°C in the dark.

2.6.2 Mating yeast cells

Cells of opposite mating type were patched onto one another and allowed to grow at 29°C for 1 to 2 days on low glucose (0.1 % (v/v) YPA media. Zygotes were selected either manually using a Singer micromanipulator or by growth on media to select for diploids.

2.6.3 Sporulation and tetrad dissection

Diploid strains to be sporulated were grown on YPAD plates overnight at 29°C. Cells were patched onto a sporulation plate and left on the bench for up to five days. The plate was monitored daily after 2 days to check the extent of development of four-spored asci.

When these were plentiful, cells were taken from the plate using a toothpick and were resuspended in 100 μ l filter sterilised 0.1 M potassium phosphate buffer pH 7.4 containing 0.5 mg ml⁻¹ 100T zymolyase (ICN Biomedical) and incubated for 10 min at room temperature. Once digested, spores were struck onto YPAD plates, placed under a micromanipulator (Singer) with the needle beneath and facing up toward the cells on the plate. Looking down the eyepiece of the micromanipulator, the region of spread cells was located and tetrads isolated and dissected into rows of four spores on the YPAD plate using the x and y co-ordinates. When sufficient spores were isolated the plate was incubated at 29 °C until the spores germinated and became visible. The number of viable spores was noted and they were patched onto YPAD or selective plates to test for genetic markers.

2.6.4 Yeast Transformation

Cells were grown to mid log phase ($A_{600} = 0.5$). 15 ml cell suspension was centrifuged at 600g for 5 min and washed once in TE (10 mM Tris/HCl, 1 mM EDTA pH 7.5) and once in 0.1M lithium acetate made up in TE. Cells were then resuspended in 100 μ l 0.1 M Lithium acetate/ TE. In a separate tube 15 μ l 10 mg ml⁻¹ herring sperm DNA, which had been boiled for 15 min was mixed with 0.1 to 1 μ g transforming DNA. This mixture was then vortexed briefly, 700 μ l 40 % (v/v) PEG4000 in TE containing 0.1 M lithium acetate was added and the mixture vortexed again. The transformation mixture was incubated at room temperature for 90 min on a mechanical rotator before being heat shocked at 42°C for 15 min. Cells were spun down in a microfuge (3000 g for 1 min) and resuspended in 100 μ l of sterile water and plated out on selective plates (see Section 2.6.1 for yeast selection media).

2.6.5 Temperature sensitivity of yeast on solid growth medium.

A single colony of cells was picked using a toothpick and was resuspended in 100 μ l water. 3 μ l of diluted yeast were added to YPAD plates and incubated at either 30°C or 37°C for 1 to 2 days to assess whether a strain had a temperature-sensitive defect.

2.6.6 Glycerol Stocks of yeast cells

For long-term storage of yeast, 1 ml culture was mixed with 1 ml 50 % sterile glycerol in a cryovial and then stored in a -80°C freezer.

2.7 Materials

All chemicals used were from Merck or Sigma unless otherwise stated.

2.8 Molecular and cell biology tools

2.8.1 Plasmid DNA

pKA280 = p416MET25 vector (Mumberg et al 1994) containing SCP1 coding sequence excised from an *EcoRI/XhoI* digestion of pSJW1-SCP1

pKA281 = p426MET25 vector (Mumberg et al 1994) containing SCP1 coding sequence excised from an *EcoRI/XhoI* digestion of pSJW1-SCP1

pSJW1-SCP1 = modified pET (Winder & Kendrick-Jones 1995) vector containing SCP1 generated by PCR amplification of the SCP1 coding sequence from genomic DNA and cloned into *NdeI/SalI* restriction sites

pGEX-4T-SCP1 = pGEX-4T vector (Amersham Bioscience) containing SCP1 generated by PCR amplification of the SCP1 coding sequence from pSJW1-SCP and cloned into *EcoRI/XhoI* restriction sites

pC-termSCP1-GST = pGEX-4T vector (Amersham Bioscience) containing SCP1 generated by PCR amplification of the C-terminal SCP1 coding sequence (142 – 200) from pGEX-SCP and cloned into *EcoRI/XhoI* restriction sites

pN-termSCP1-GST = pGEX-4T vector (Amersham Bioscience) containing SCP1 generated by PCR amplification of the N-terminal SCP1 coding sequence (1 – 142) from pGEX-SCP and cloned into *EcoRI/XhoI* restriction sites

pC-termSCP1-HIS = pET14b vector (Novagen) containing SCP1 generated by PCR by PCR amplification of the C-terminal SCP1 coding sequence (142 – 200) from pSJW1-SCP and cloned into *NdeI/XhoI* restriction sites

pGFP-SCP1 = This plasmid was generated by Dr K. Ayscough (Winder, Jess, & Ayscough 2003). An N-terminal GFP tagged SCP1 was generated by PCR of SCP1 from genomic DNA

2.8.2 Oligonucleotides

Okaa85F:- 5'- SCP1 oligos for GST fusion in pGEX4T:

GAT CGG ATC CAT GAG TTA CGA TAA GAA G
BamHI site(underline)

Oka86R:- 5'- CTA GCT CGA GAA CTA TAT CTC TCC GTT G

Oka254F:- 5'- For amplifying C-term. of Scp1

GAT GAA TTC CAT ACA GAT AGA TTT CCA GGT-3'

Oka255R:- 5'-CTA GCT CGA GTT ATT TTT TGT TGG CGT AAC GAG-3'

Oka338:- 5'- GCC AAC CAT ATG /CAT ACA GAT AGA TTT CCA GTT
Slash position indicated 418 and 438 of Scp1

2.8.3 Yeast Strains

KAY446 haploid α his3 Δ 1, leu2 Δ , ura3 Δ , met15 Δ

KAY448 haploid α his3 Δ 1, leu2 Δ , ura3 Δ , met15 Δ , Δ Scp1::KanMx

KAY449 haploid α his3 Δ 1, leu2 Δ , ura3 Δ , lys2 Δ , Δ Scp1::KanMx

KAY337 α , Δ arg6::LEU2, Δ pdr5::TRP1, Δ sng2::hisG, ura3-52, lys2-801(oc), ade2-101(am), trp1- Δ 63, his3 Δ 200, leu2- Δ 1.

KAY348 haploid α , trp1-1 (am), his3 Δ 200, leu2-3,112, ura3-52, lys2-801(oc), sac6::URA3, heat sensitive.

KAY349 haploid α , trp1-1(am), his3 Δ 200, leu2-3,112, ura3-52, lys2-801(oc), sac6::URA3, sensitive.

KAY115 haploid α , rho⁺, ade2-101 (am), his3-11, 15 leu2-3,112 trp1-1(am) ura3-1 cap2- Δ 1::HIS3

KAY604 KAY447 transformation with pKA256 (empty vector) MAT α his3 Δ 1 leu2 Δ lys2 Δ ura3

KAY646 KAY446 (Research Genetics BY4741) MAT α his3 Δ 1 leu2 Δ met15 Δ ura3 Δ , pKA280+Scp1p (EcoR1/Xho1)

KAY647 As above but pKA281+ Scp1p (EcoR1/Xho1), overexpressing Scp1.

3. Results

3.1 Purification of Scp1p

3.1.1 Expression of Scp1p

The vector pSJW1- SCP1 was transformed into the *E. coli* strain BL-21 (DE3) (Section 2.1.8); this strain has been extensively used in protein overexpression due to its absence of proteases. Scp1p was cloned downstream of the β -Gal promoter and IPTG was used to inhibit the suppression of this promoter and as a result drive protein synthesis. Fig. 3.1a shows protein samples from before and after induction with a band of around 22 kDa being heavily stained after induction, the presence of this band in the pre- induction sample is due to leakage through the β -Gal promoter.

After the cells had been lysed the Scp1p was found as an insoluble protein (Fig. 3.1b) aggregated in inclusion bodies; these were then isolated from the other cellular material by a series of washes (Fig. 3.1b) (Section 2.2.2), before suspending in urea for further purification.

3.1.2 Purification of Scp1p: ion exchange chromatography

In order to purify Scp1p from the other proteins in the inclusion bodies it was necessary to use various column chromatography techniques. In the first instance it was decided to use ion exchange chromatography (Section 2.2.3); this separates proteins using their affinity to bind to either a positive or negative charged resin. Scp1p has a predicted pI of 9.5 and therefore would be expected to bind to a cation exchanger resin at pH values near neutrality.

The weak cation exchanger CM-Sepharose-CL6B had previously been used in the purification of calponin (Winder and Walsh, 1990) and this proved an effective first step in the purification of Scp1p. It readily bound to the resin with displacement occurring at salt concentrations of

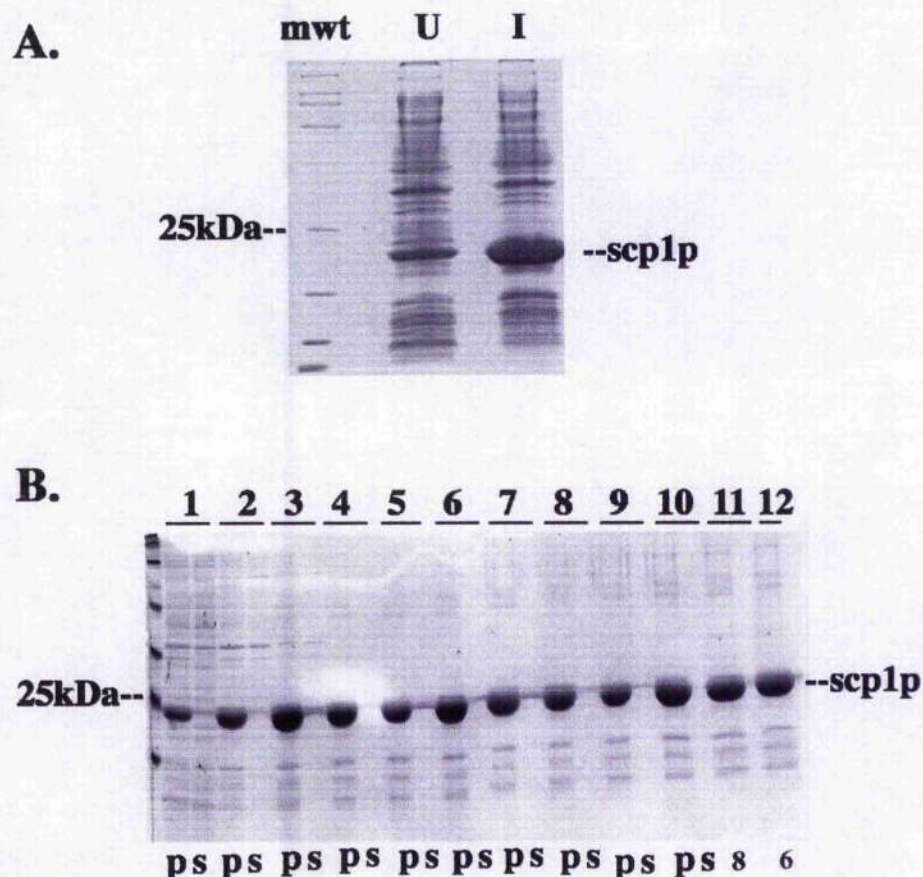


Fig 3.1. Expression of Scp1p and isolation of Scp1p-rich inclusion bodies: (A) Protein samples were taken from BL-21 *E. coli* containing pSJW 1-SCP1 both before (u); and after (i) induction and separated on a 12.5% poly-acrylamide gel, a band of ~22 kDa corresponding to Scp1p can be seen to increase in intensity after induction. (B) Scp1p is absent in the soluble fraction of protein (s1) but is found in the pellet (p1) as inclusion bodies. Purification of these inclusion bodies was achieved by carrying out a series of wash/low speed centrifugation steps (2 – 10), with samples from each pellet (p) and supernatant (s) fractions analysed. Scp1p containing inclusion bodies were then solubilised in 8 M urea (11) and then diluted down to 6 M urea (12) ready for purification.

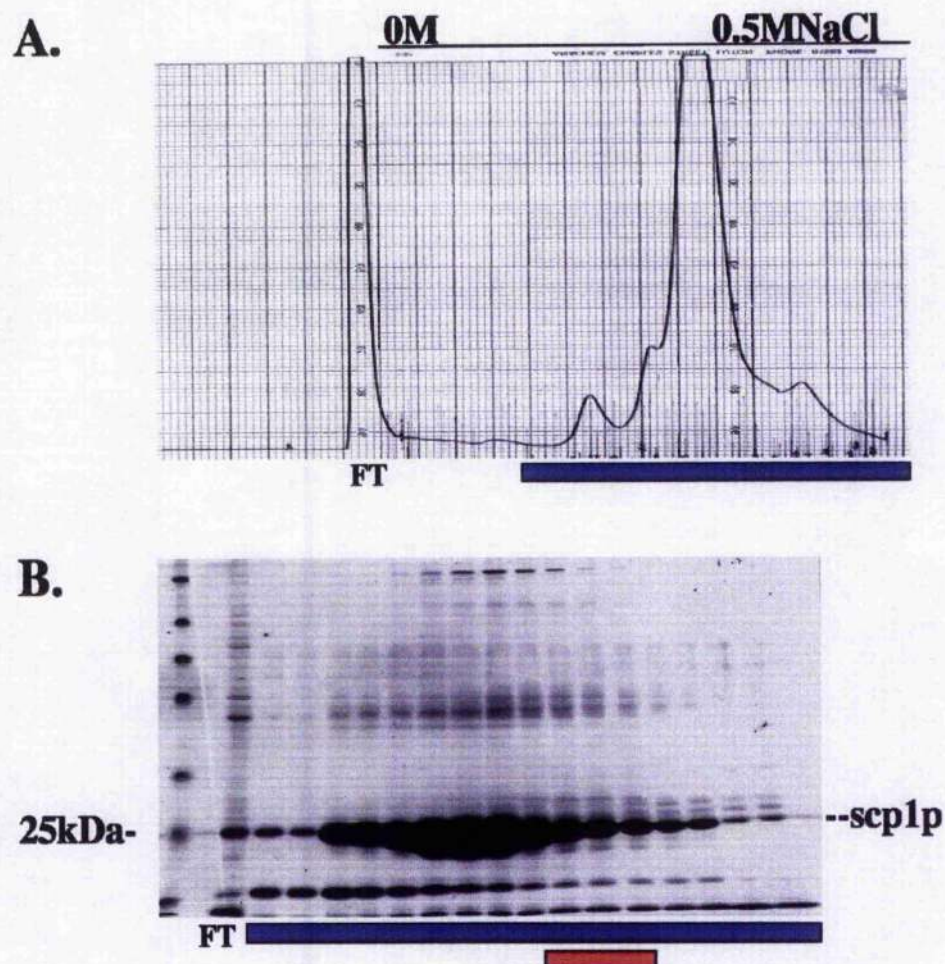


Fig. 3.2 Purification of Scp1p by ion exchange chromatography

Scp1p-containing inclusion bodies solubilised in 6 M urea were loaded onto a CM-CL6B ion exchange column, and eluted using a 0-500 mM NaCl gradient. The blue bar indicates the fractions which were eluted at ~200 mM NaCl and were analysed by SDS-PAGE. (B) Analysis of fractions from ion exchange on SDS-PAGE shows a separation from some of the contaminating bands. The red bar indicates the fractions which were considered to be the least contaminated by the high and low molecular mass species, and were then pooled together for further purification

around 200 mM (Fig. 3.2a). Since more than one protein was present in the all of the fractions comprising the peak, it was necessary to select the ones which contained the fewest contaminants (Fig. 3.2b), for use in further purification steps.

3.1.3 Purification of Scp1p: hydroxyapatite chromatography

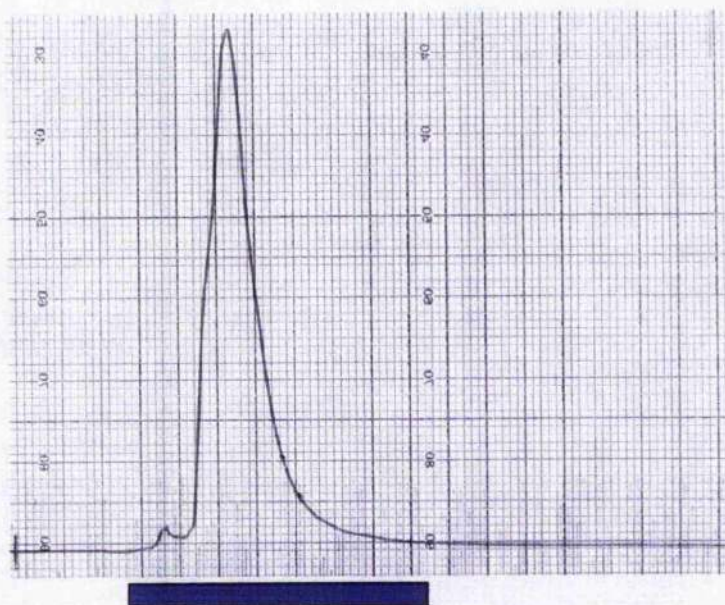
Other purification methods which have been used on proteins of the Scp1p family include gel filtration chromatography (Section 2.2.4); this was ruled out due to the presence of contaminating proteins close to the molecular mass of Scp1p, and hydroxyapatite chromatography (section 2.2.5). Hydroxyapatite is a crystalline form of calcium phosphate $[\text{Ca}_5(\text{PO}_4)_3\text{OH}]_2$ which, because it contains both positively and negatively charged centres, can operate as a mixed ion-exchanger. Hydroxyapatite can be used in both batch and column modes, and is chemically and thermally stable with a low non-specific adsorption of hydrophobic macromolecules. Because elution is generally performed under mild conditions, hydroxyapatite chromatography generally gives good recoveries of proteins.

Scp1p was found to bind to hydroxyapatite and elution occurred at a phosphate concentration of around 20mM (Fig. 3.3a). Although there were still small amounts of contaminating proteins present, some of the fractions were deemed to be pure enough (>95%) for further experimental purposes (Fig. 3.3b).

3.1.4 Refolding Scp1p from urea

In order to carry out functional analysis of Scp1p it was necessary to convert the protein to its native form, since the purification steps had been carried out on the protein in a denatured state in 6 M urea. Refolding of the Scp1p involved a step-by-step dialysis from urea into a suitable buffer, which should allow folding to occur. The correct conditions were established by performing test experiments using buffers with a range of pH (6.0, 6.5, 7.0, 7.5, 8.0, 8.5, & 9.0) and NaCl concentrations (0, 50, 100, 25, & 500 mM). The only conditions under which Scp1p remained soluble during the dialysis from urea were pH 6.0 (this was achieved using 20 mM Mes pH 6.0) with 0 mM NaCl. Even under these

A.



B.

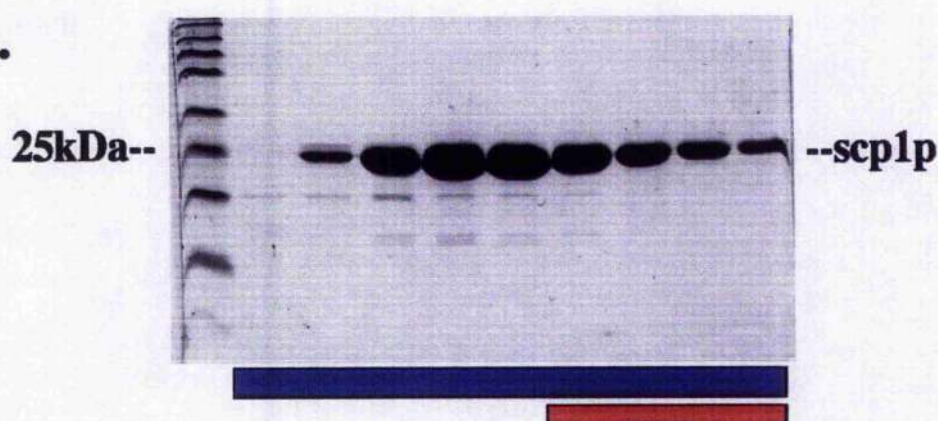


Fig. 3.3 Purification of Scp1p by hydroxyapatite chromatography

In (A) Scp1p containing fractions pooled together from ion exchange chromatography were loaded onto a hydroxyapatite column, and eluted using a 1-100 mM KH_2PO_4 gradient; the blue bar indicates the fractions analysed by SDS-PAGE. (B) Analysis of fractions by SDS-PAGE shows a separation of scp1p from contaminating proteins; the red bar indicates the fractions considered to be the least contaminated by the high and low molecular mass species, which were then pooled together.

conditions which allowed refolding, less than half of the total Scp1p remained soluble after the complete removal of the urea. It should be noted that in order to concentrate Scp1p after refolding, it was necessary to use a pressure-driven concentrator instead of the centrifugation type as use of the latter caused the Scp1p to form a gel in the bottom of the concentrator tube.

3.1.5 CD Analysis of purified Scp1p

Once purified Scp1p had been obtained, it was necessary to establish that it was indeed correctly folded; this was done using the technique of Circular Dichroism (CD). CD exploits the fact that different secondary structural features in a protein possess chirality and these will absorb left and right circularly polarised components of polarised light differentially. The resultant trace (Fig. 3.4) in the far UV region where the peptide bonds absorb can then be analysed using various published programs to estimate the secondary structure composition of a protein. Previous investigations on proteins containing single CH Domains have shown them to possess a significant amount of α -helix and Scp1p appears to fit this pattern with a predicted secondary structure consisting of: 23 % α -helix, 19 % anti-parallel sheet, 4 % parallel sheet, 21 % turns, and 33 % other structures. It was also found that pH had a marked effect on the stability of the purified Scp1p with a shift from pH 6.0 to pH 8.0 resulting in a greater degree of unfolding of the protein than was obtained with 4 M urea (Fig. 3.4).

3.2 Actin-binding properties of Scp1p

3.2.1 Characteristics of Scp1p binding to actin

In order to determine whether the purified Scp1p could bind to actin, a high-speed sedimentation assay (100000 g) was performed (Section 2.3.3). The result from this (Fig. 3.5a) shows that the Scp1p alone is present in the supernatant fraction and migrates to the

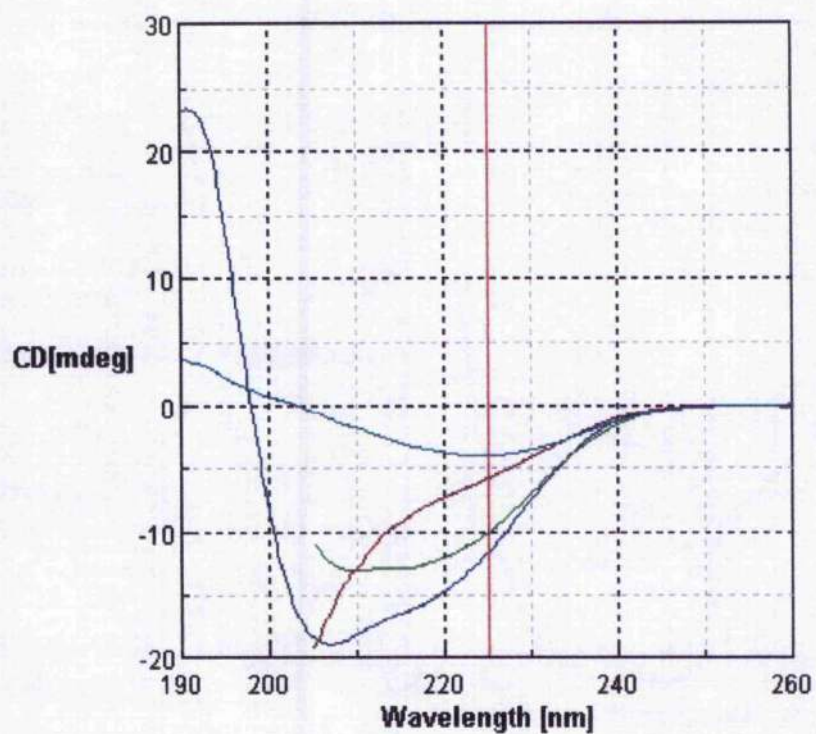


Fig. 3.4 Far UV CD spectra of Scp1p

CD measurements were performed on a 0.5 mgml⁻¹ Scp1p solution containing:- 20 mM Mes pH 6.0, 1 mM EGTA (blue); 20 mM Mes pH 6.0, 1 mM EGTA, 2 M GdnCl (green); 20 mM Mes pH 6.0, 1 mM EGTA, 4 M GdnCl (red); 10 mM Tris pH 8.0, 1 mM EGTA, (aqua), using a cell with a 0.02 cm pathlength.

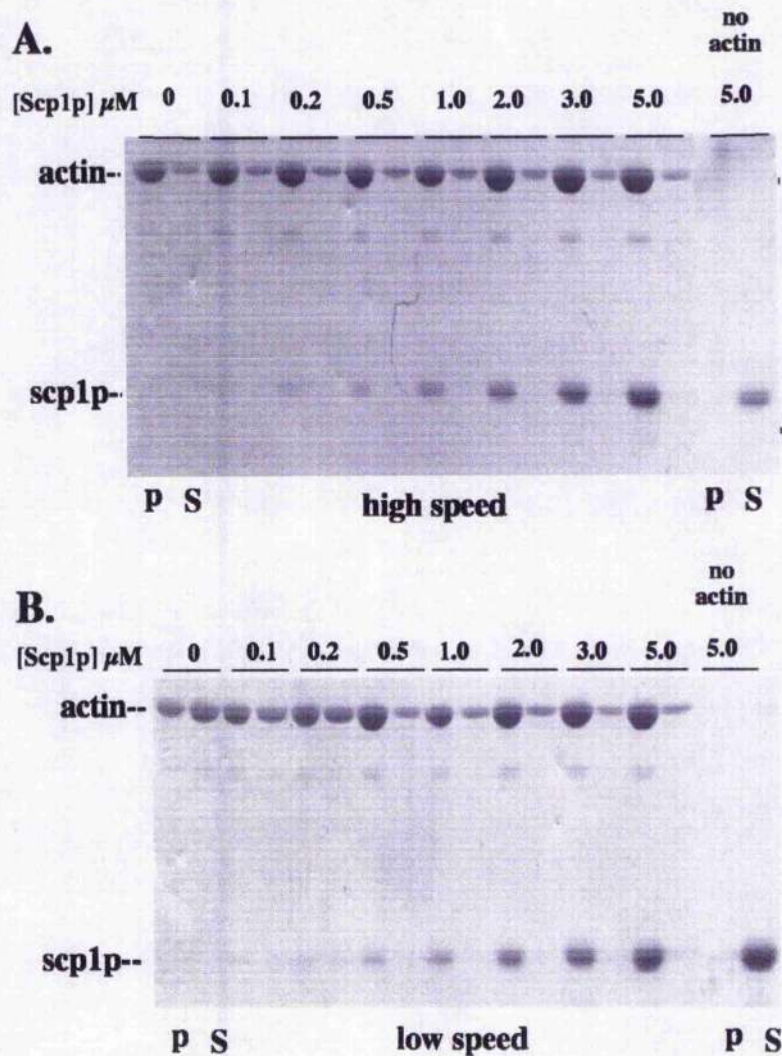


Fig. 3.5 Sedimentation assay for actin binding

(A.) Increasing concentrations of Scp1p, from 0.1 to 5 μ M were incubated with 5 μ M F-actin and subjected to high speed ultracentrifugation. Scp1p is seen to co-sediment with actin in the pellet (P) whereas Scp1p alone remains in the supernatant (S) (B) F-actin was subjected to low-speed sedimentation after polymerisation in the presence of increasing concentrations of Scp1p. In the absence of Scp1p most of the actin is found in the supernatant, however after addition of at least 0.1 μ M Scp1p more actin co-sedimented in the pellet with Scp1p.

Pellet fraction when mixed with F-actin, indicating its ability to bind to actin. Calponin and SM22, proteins which are both homologous to Scp1p have been shown to induce the formation of parallel bundles of actin filaments (bundling) or cross-link actin filaments into mesh-like structures (gelling) (Kolakowski et al 1995, Shapland et al 1993) and therefore it was predicted that Scp1p might have a similar activity. To investigate this, a low speed sedimentation assay was performed (Section 2.3.2); the slower centrifugation (13000 g) pellets actin bundles whilst leaving unbundled F-actin in the supernatant. The experiment shows (Fig. 3.5b) that with increasing concentrations of Scp1p, there is a clear shift in the actin from the supernatant to the pellet fraction.

The different effects that actin-binding proteins can have on F-actin can also be monitored using a falling ball viscosity assay (Section 2.3.1), which exploits the change in viscosity of a solution of G-actin as it is polymerised to F-actin. A further change in viscosity occurs when F-actin is either cross-linked, bundled or severed. Since the results of the low speed sedimentation assay pointed to a role for Scp1p in bundling actin filaments, it was expected that an increase in viscosity would occur as a meshwork of actin bundles was created. Surprisingly, when Scp1p was added to 5 μ M actin in this assay a decrease in the viscosity was observed (Fig. 3.6), with a >95 % decrease in viscosity occurring at as little as 1 μ M Scp1p. Although the viscosity results appeared to contradict the sedimentation data it should be noted that other bundling proteins such as Scrui (Owen et al 1993, Schmid et al 1994) exhibited the same characteristics. The observations could be explained by Scp1p inducing the formation of very tight parallel bundles, which could then be easily displaced by the ball bearing in the viscosity assay. In order to visualise the F-actin structure, Scp1p was added to actin and polymerised before being negatively stained and viewed by electron microscopy (Fig. 3.7) (Section 2.3.4). It can be seen from Fig. 3.7b that Scp1p does indeed cause the formation of tight actin bundles as they are absent from Fig. 3.7a which shows actin alone.

3.2.2 The effect of Scp1p on the kinetics of actin polymerisation/ depolymerisation

The kinetics of actin polymerisation/depolymerisation have been widely used to characterise actin-binding proteins. This technique exploits the change in fluorescence of pyrene-labelled G-actin as it is converted to F-actin. Scp1p was added to a solution of

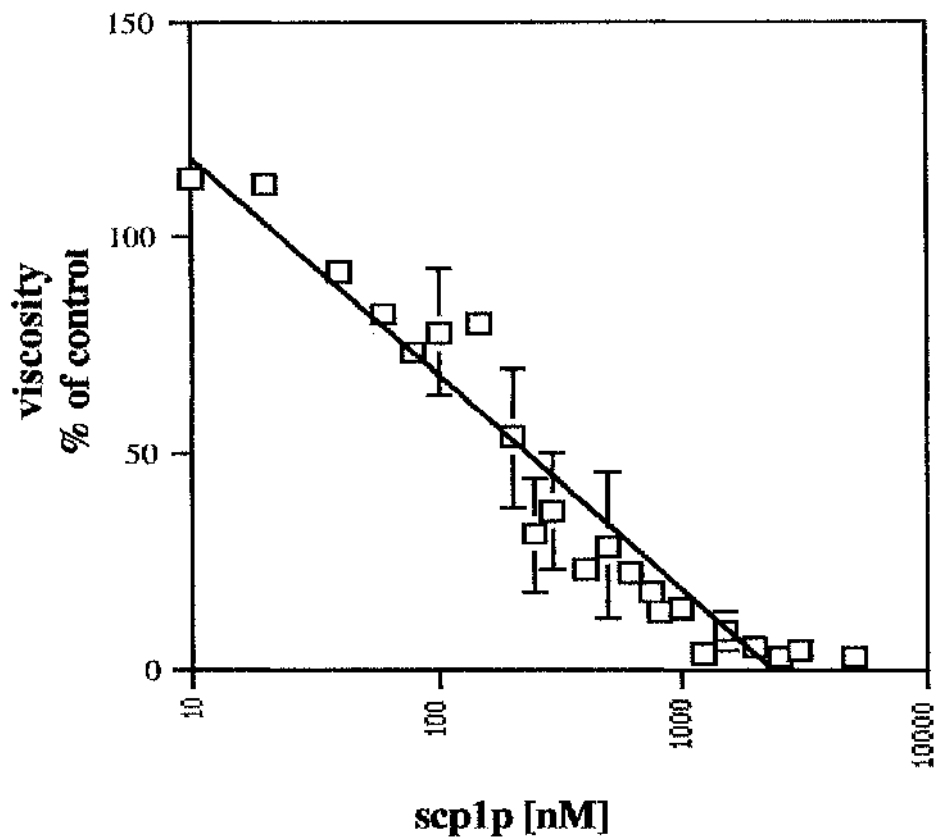
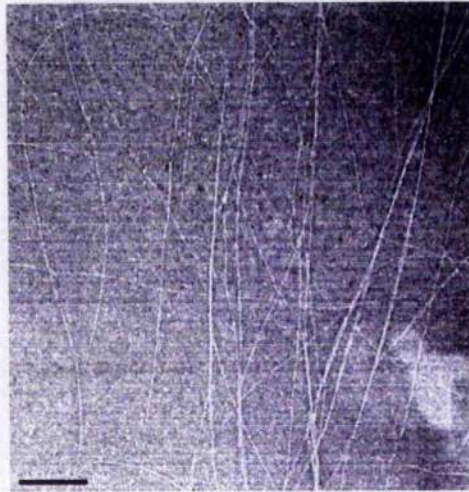


Fig. 3.6 Falling ball assay viscosity assay

Falling -ball assays were performed to investigate the effect of Scp1p on the viscosity of an F-actin solution. Increasing concentrations of Scp1p resulted in a decrease in viscosity of the solution, indicating either a severing or very tight bundling activity.

A.



B.

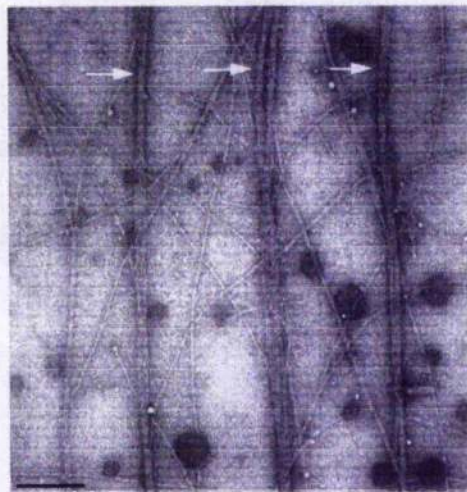


Fig. 3.7 Electron microscopy of actin and Scp1p

Actin filaments were polymerised in the absence (**A**) or presence (**B**) of 1 μM Scp1p for 3 h. Filaments were negatively stained with Nanovan (Section 2.3.4) and visualised using a Zeiss 902 electron microscope. The scale bars represent 200 nm. Arrows indicate Scp1p-induced actin bundles.

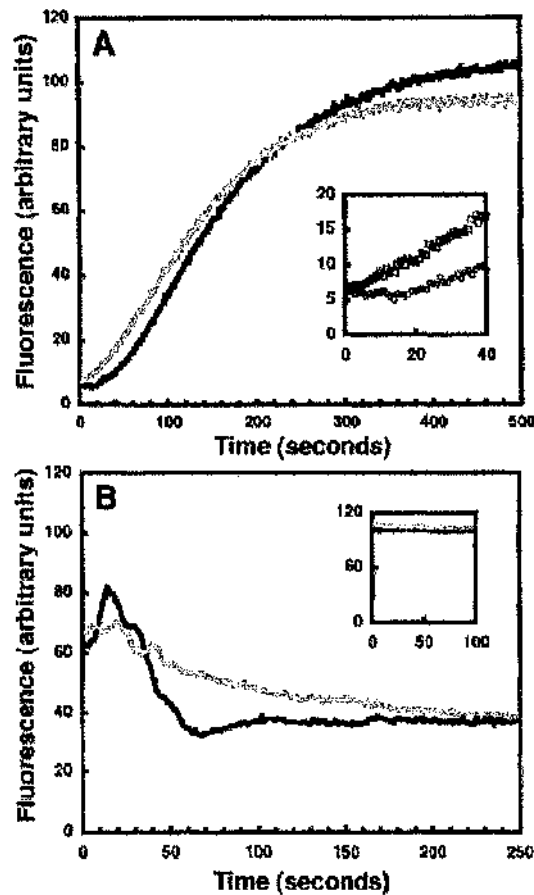


Fig. 3.8 Effects of Scp1p on polymerisation/depolymerisation kinetics of pyrenyl-actin: 1 μM pyrene-actin was polymerised in the absence (black line, circles on inset) or presence (grey lines, squares on inset) of Scp1p (0.5 μM) and the resulting fluorescence measured over 1000 s (panel A). There was no apparent difference between these two samples except for a slight reduction in the lag phase, which is further illustrated in the expanded view which covers the first 40 s (inset box). Depolymerisation kinetics (panel B) of pyrene-actin plus Scp1 (grey line) were also slightly retarded compared with those of pyrene-actin alone (black line). There was no change in the steady state kinetics of pyrene-actin turnover (panel B inset) in those samples containing pyrene-actin plus Scp1p (grey line), compared with those containing pyrene-actin on its own (black line).

pyrene-actin and polymerised (Section 2.3.5). As shown in Fig. 3.8 there was no effect on the rate of polymerisation except for a slight reduction in the lag phase; this can be ascribed to the stabilisation of short filaments due to bundling. A slight inhibition of depolymerisation could also be observed which is also common amongst actin-bundling proteins reflecting the increased stability of the F-actin.

3.2.3 The regions of Scp1p which are responsible for actin-binding and bundling activity

It has been previously reported that the actin-binding domain of calponin does not include the amino terminal CH domain but starts on its C-terminal portion and includes the first calponin-like repeat (CLR) (Gimona, 1998). In order to determine whether this was also the case for Scp1p, a 6 kDa fragment was generated by limited proteolysis of the full length protein and used in a high speed sedimentation assay (Section 2.3.3). As shown in Fig. 3.9, a 6 kDa band is found in the supernatant in the absence of actin, which shifts to the pellet as it is added to actin. Mass spectrometry (MS) of this fragment yielded a major species with mass of 6297 Da, which was predicted to correspond to residues 139-195 of Scp1p. Internal sequencing of this fragment carried out by MS yielded the sequence YGYMKGASQATEGVVLGQ corresponding to residues 177-194 of Scp1p and confirming its identity as the C-terminal 60 residues of Scp1p including the CLR but excluding the CH-domain.

To define in more detail the regions of Scp1p which are responsible for actin binding, it is necessary to generate truncation mutants. To generate N- and C-terminal fragments, forward and reverse primers were designed complementary to the coding sequences of the N-terminal (amino acids 1-142), and C-terminal (amino acids 142-200) parts of Scp1, and were engineered to have EcoR1 and Xho1 restriction sites in the forward and reverse primers to allow further sub-cloning. The PCR-amplified fragments of the predicted size (ie. 400 bp for the N-terminal mutant and 200 bp for the C-terminal) mutant were then ligated into the EcoR1/Xho1 sites of the expression vector PGEX-4T (Fig. 3.10). A third cDNA corresponding to full length Scp1 (amino acids 1-200) was also cloned into this vector using this strategy (Fig. 3.10c discussed later in Section 3.3.3). Clones were sequenced to check for errors and expression of the mutant protein tested (Fig. 3.11).

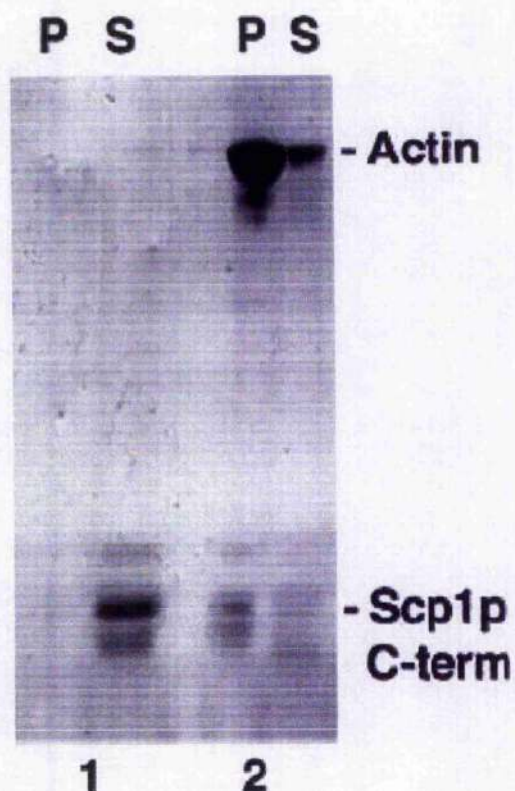


Fig. 3.9 Analysis of proteolytic fragment of Scp1p reveals an actin-binding region: Proteolytic fragments of Scp1p (5 μ M) were subjected to ultracentrifugation, after incubation in the absence (lanes 1), or presence (lanes2) of actin (5 μ M). After analysis by SDS-PAGE using a 7.5-20 % , a 6 kDa band; corresponding to the C-terminal region of Scp1p, can be seen to shift from the supernatant (S) fraction to the pellet (P) fraction with actin.

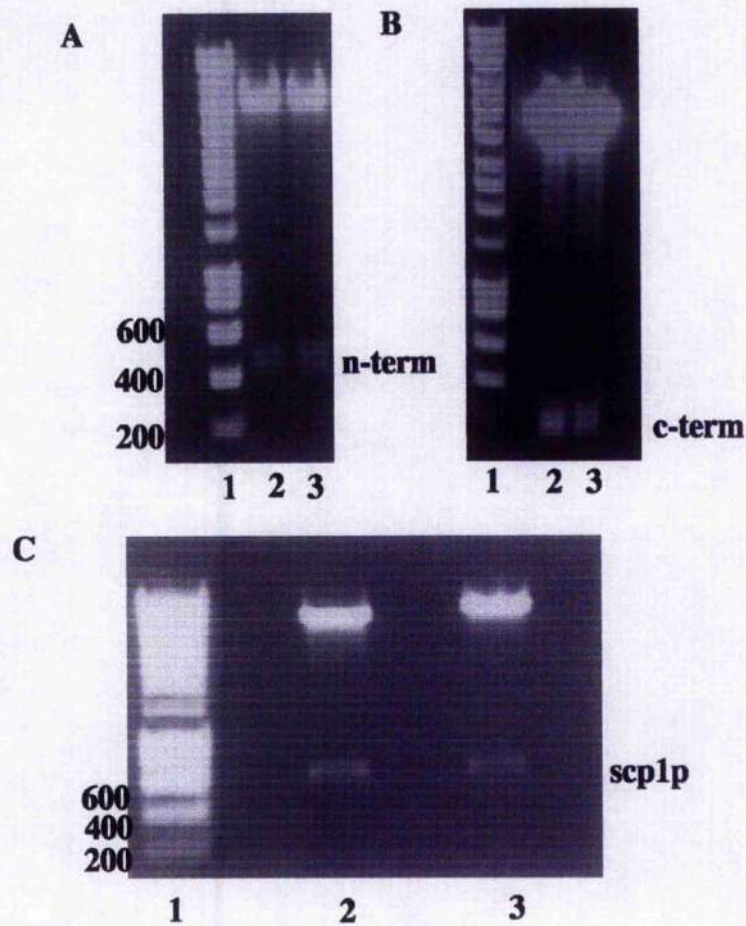


Fig. 3.10 Restriction analysis pGEX-4T clones, containing SCP1

fragments: (A) The CH domain of SCP1 was cloned into pGEX-4T, and checked by an EcoR1/Xho1 restriction digest, before being separated on a 1 % agarose gel. The presence of a band of ~400 bp, corresponding to the CH domain of SCP1 can be seen in both clones 2 and 3. (B) The CLR of SCP1 was cloned into pGEX-4T, and checked by an EcoR1/Xho1 restriction digest. The presence of a band of ~200 bp, corresponding to the CLR of SCP1 can be seen in both clones 2 and 3. (C) Full length SCP1 was cloned into pGEX-4T, and checked by an EcoR1/Xho1 restriction digest. The presence of a band of ~600 bp, corresponding to full length SCP1 can be seen in both clones 2 and 3.

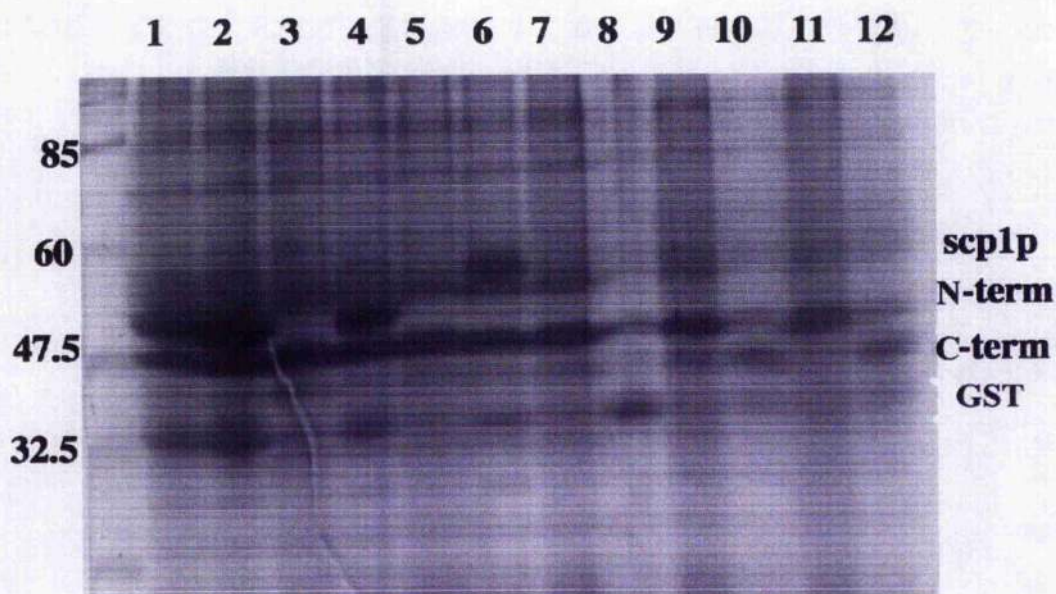


Fig. 3.11 Expression of Scp1p and its N- and C-terminal mutants expressed as GST-fusion proteins.: BL-21 (DE3) E.coli were transformed with pGEX-4T N-term SCP1 (lanes 1-4); pGEX-4T SCP (lanes 5 and 6); pGEX-4T (lanes 7 and 8); and pGEX-4T C-term SCP1 (lanes 9-12). Cells containing these plasmids were then grown to mid log phase, before expression was induced, and a sample removed before (lanes 1, 3, 5, 7, 9, 11); and after (lanes 2, 4, 6, 8, 10, 12) induction. Samples were then analysed by SDS-PAGE on a 12.5 % gel.

Purification of the mutants and full length GST-fusion protein was carried out according to the manufacturers recommendations (section 2.2.10); however the C-term-GST fusion did not express a soluble protein (data not shown) and so a different strategy was adopted. The C-terminal CLR region was amplified from pSJW1-Scp1p using primers containing engineered NdeI and XhoI restriction sites which would allow cloning into a pET-14B vector at the appropriate sites to generate a 6xHis-tagged over-expressed protein. This was then purified according to the manufacturers recommendations (section 2.2.10)-9+. High speed sedimentation assays (section 2.3.3) with the soluble Scp1p mutant proteins (Fig. 3.12) shows that a significant proportion of the C-terminal His-tagged mutant is present in the pellet fraction, indicating a role for the CLR of Scp1p in actin binding. The GST-N-term mutant does not co-sediment with actin in this assay, consistent with previous work showing that CH domains are not sufficient on their own to bind actin (Gimona & Winder 1998).

3.3 *In vivo* studies of the role of Scp1p in *S. cerevisiae*

3.3.1 Co-localisation of Scp1p with actin

In order to understand the role that of Scp1p within the cell in more detail an antibody was raised against the protein after purification on SDS – PAGE (section 2.2.6). Following affinity purification (section 2.2.9) the antibody was able to cross-react with a protein of mass around 20 kDa, corresponding to Scp1p, in yeast whole cell lysates (Fig 3.13).

This antibody was then used in immunofluorescence microscopy (section 2.4.2), to examine the intracellular localisation of Scp1p within the yeast cell, and in particular whether it is co-localised with actin. However, despite using a variety of fixation techniques and processing procedures no specific staining for scp1p could be detected. Since the biochemical evidence (section 3.2) points to a role for Scp1p in the formation of tight actin bundles, the lack of detection by the antibody could arise from a blocking of the epitopes on the protein due to its presence within the actin bundles.

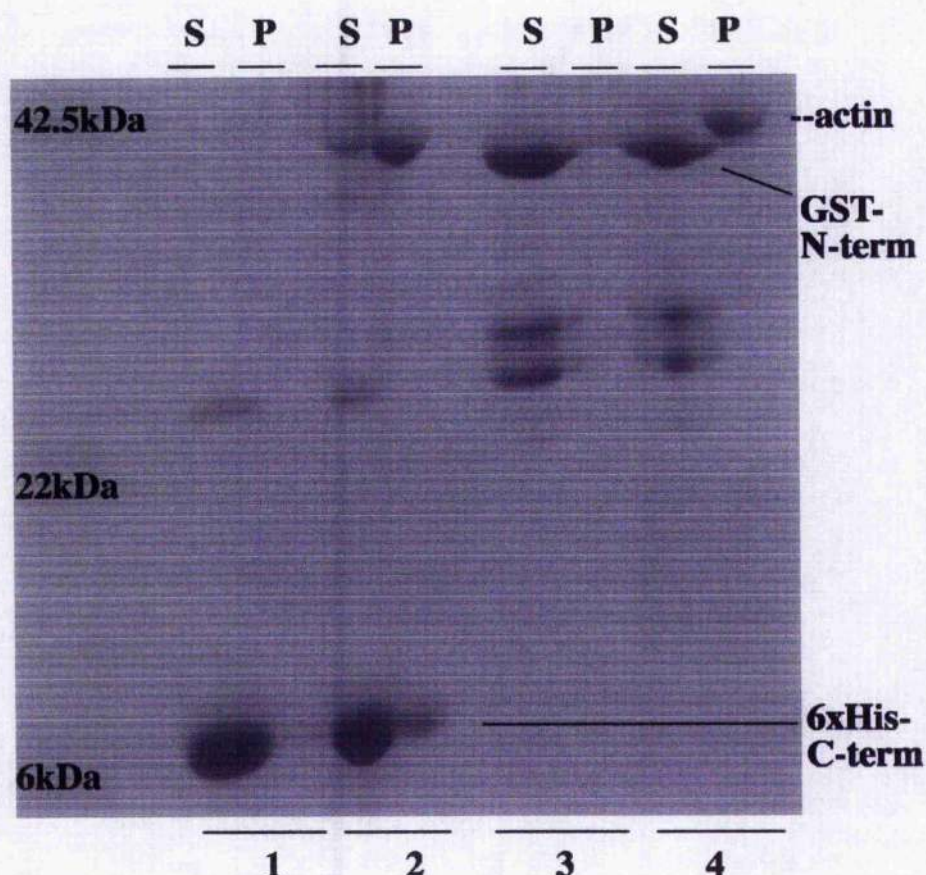


Fig. 3.12 Actin-binding properties of Scp1p fragments.

6xHis-taggedC-term Scp1p (25 μ M lanes 1 and 2) or, GST N-term scp1p (10 μ M lanes 3 and 4) was incubated in the absence (lanes 1 and 3), or presence (lanes 2 and 4) of actin (5 μ M) and subjected to ultracentrifugation followed by analysis by SDS-PAGE on a 15 % gel. A significant amount of the 6xHis-taggedC-term Scp1p can be seen to shift from the supernatant to the pellet fraction in the prescence of actin, whereas there was no change in the distribution of GST N-term scp1p. Proteins of interest are labelled, with the remaining bands consisting of a co-purifying yeast protein (lanes 1-4), and a degradation product (lanes 3 and 4).

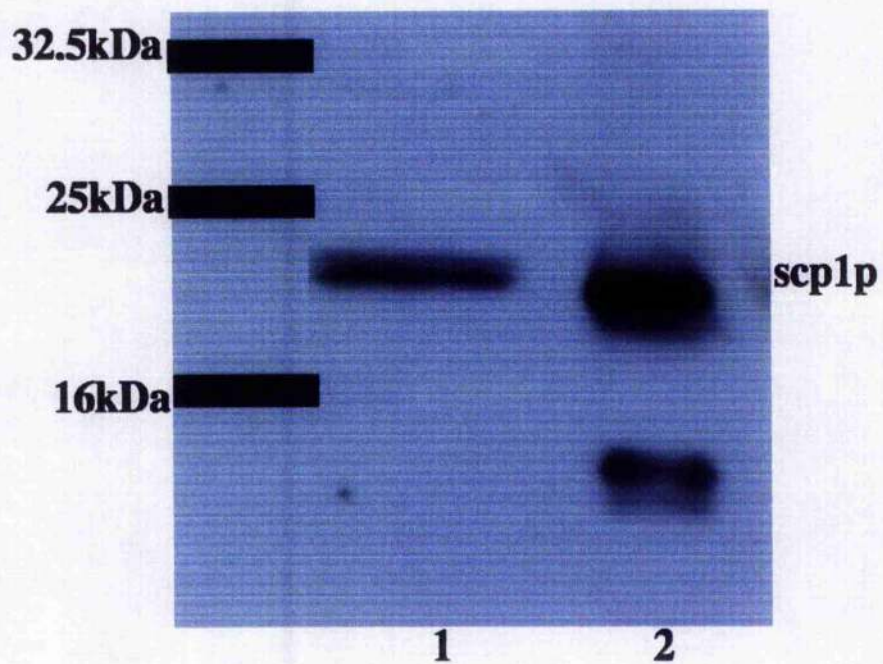


Fig. 3.13 Immunoblotting of Scp1p

Western blot analysis using an affinity purified anti-Scp1p rabbit polyclonal antibody detects a band of ~22 kDa in a wild type yeast, whole cell lysate (lane 1) which runs parallel to purified Scp1p (lane 2). There was some degradation of the purified Scp1p, resulting in the presence of a lower band in lane 2.

In order to visualise the intracellular location of Scp1p it was tagged with Green Fluorescent Protein GFP at its N-terminus (Winder, Jess, & Ayscough 2003). Immunofluorescence experiments using GFP-Scp1p along with rhodamine-phalloidin staining revealed a co-localisation of Scp1p with actin patches at the cell cortex (Fig. 3.14). This co-localisation of Scp1p to cortical actin patches can also be seen to be disrupted in cells expressing GFP-Scp1p which were exposed to the actin monomer-sequestering drug latrunculin-A; this is consistent with Scp1p having a role in the formation or stabilisation of cortical actin patches (Winder, Jess, & Ayscough 2003).

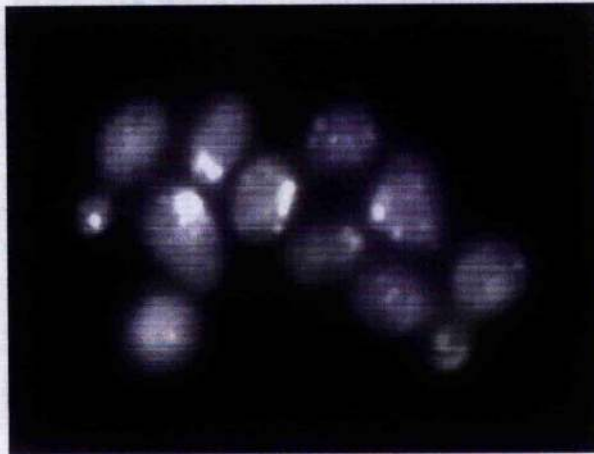
3.3.2. Effect of deletion of SCP1 gene

In the first instance it was decided to look at the effects of deleting the SCP1 gene from *S. cerevisiae* since deletion studies in yeast have proved to be a powerful tool in helping to determine the *in vivo* role of actin-binding proteins (Adams, Botstein, & Drubin 1991); in addition gene-deleted strains are commercially available.

Microscopy using rhodamine-phalloidin (section 2.4.1) to visualise actin failed to show any differences in the organisation of the actin cytoskeleton between the SCP1-null and the wild type strains, with both actin cables and cortical actin patches being clearly visible and highly polarised at various points in the cell cycle (Fig. 3.15).

The failure of SCP1 deletion to have any effect on the actin cytoskeleton arrangement is indicative of an overlapping function with another gene. In order to characterise further the role of Scp1p *in vivo*, it was decided to examine the possible involvement of 2 other proteins, namely:- (a) Sac6p, the yeast homologue of fimbrin, the only other protein found to date shown to have a major role in actin bundling and filament stabilisation (Adams, Botstein, & Drubin 1989; Adams, Botstein, & Drubin 1991), and (b) Cap2p, an actin-capping protein, since deletion of both the CAP2 and SAC6 has been shown to be synthetically lethal (Adams, Cooper, & Drubin 1993). SCP1-null yeast cells were crossed with cells disrupted in either the SAC6 or CAP 2 gene to check for synthetic lethality (Section 2.6.2 and 2.6.3). Cells containing both sets of deletion were found to be viable (Fig. 3.16a). However it was found that cells with the $\Delta scp1 \Delta sac6$ genotype had an aberrant actin phenotype with only 2 % of them containing polarised cortical actin

A.



B.

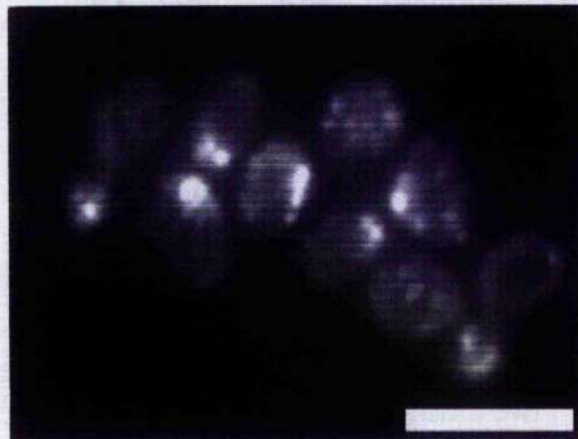
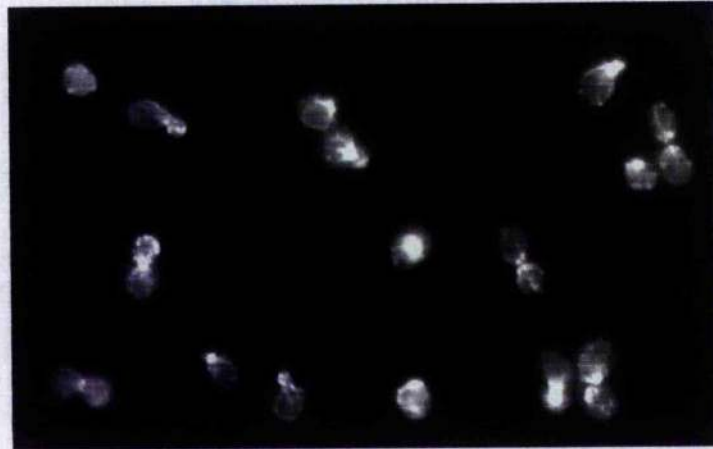
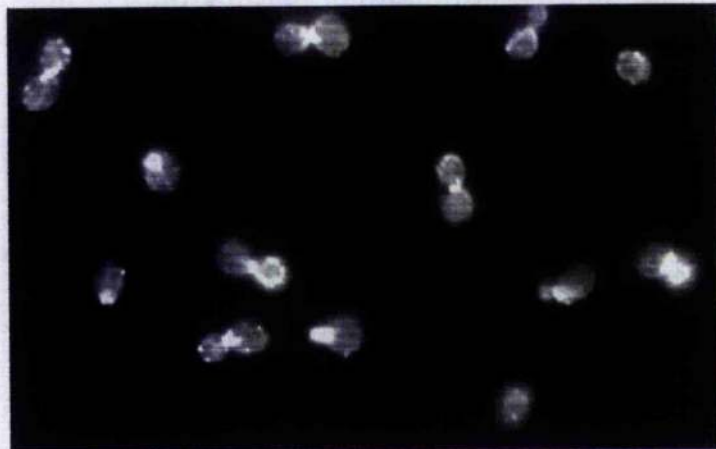


Fig. 3.14 Co-localisation of actin with Scp1p in *S. cerevisiae*

GFP-Scp1p expressing yeast cells were fixed and stained with rhodamine phalloidin to visualize actin. In panel A, GFP-Scp1p is seen to be present in small punctate patches at the cell cortex where it appears to co-localise with actin, shown in panel B stained with rhodamine phalloidin. Scale bars, 5 μm .



KAY 446



KAY 448

Fig 3.15 Effect of deletion of SCP1 gene on the organization of actin in *S. cerevisiae*:Yeast cells in which SCP1 had been deleted (KAY446) displayed no aberrant actin phenotype compared to that of wild type cells (KAY448) when viewed after fixation and staining with rhodamine-phalloidin.

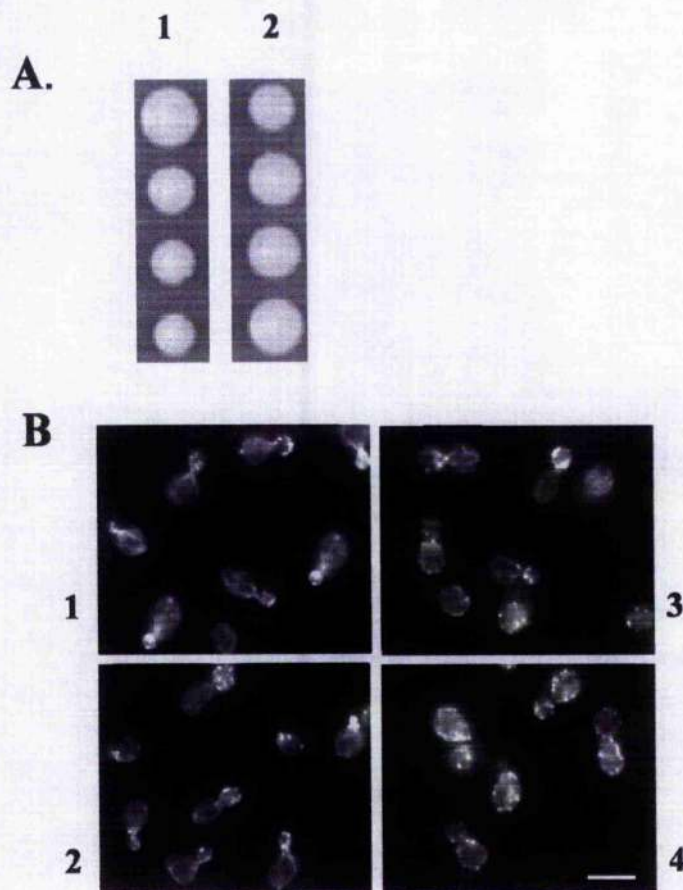


Fig. 3.16 Effects of deletion of SCP1, SAC6 and CAP2 genes in *S. cerevisiae*: (A) Yeast cells deleted for SCP1 were crossed with either cells deleted for SAC6 (panel 1) or CAP2 (panel 2) and tetrads separated from the resulting four-spored asci. These were plated out onto YPAD and left to germinate. In both panels each of the 4 spores from the tetrad produced viable colonies, indicating absence of any synthetic lethality between SCP1 and either SAC6 or CAP2. (B) Each of the four genetic variants from the cross between Δ SCP1 and Δ SAC6, were allowed to germinate and grow. After fixing, cells were stained with rhodamine-phalloidin to visualise the actin structures within. Cells with the single SCP1 deletion have only minor defects in their actin cytoskeleton when compared to cells containing both genes, with Δ SAC6 and Δ SCP1/ Δ SAC6 (panel 4) displaying an increasingly disrupted actin cytoskeleton. Scale bars, 5 μ m.

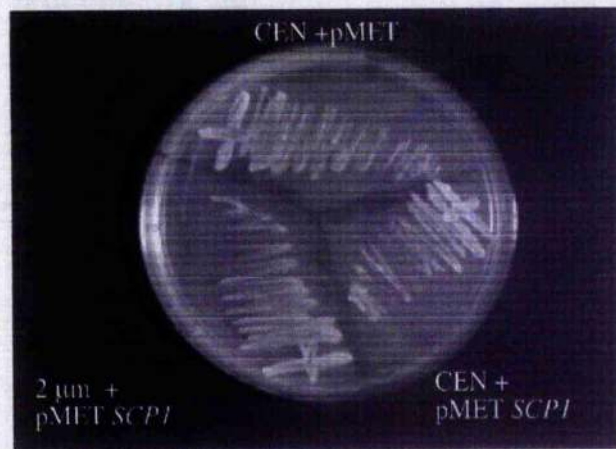
patches compared with 88 % in the $\Delta scp1$ cells and 90 % of wild type (Fig. 3.16b). Later experiments showed that the $\Delta scp1 \Delta sac6$ double mutant is more sensitive to latrunculin-A than either the $\Delta scp1$ or the $\Delta sac6$ mutants alone (Winder, Jess, & Ayscough 2003). These results suggest a role for both Scp1p and Sac6p acting together in organising the yeast F-actin cytoskeleton.

3.3.3 Effect of over-expression of SCP gene

With the Scp1p deletion mutants showing no real phenotype unless present in a SAC6 null background (Section 3.3.2) it was decided to examine whether over-expression of the SCP1 gene would have any effect on the actin cytoskeleton, since this has also been the case for the actin binding protein ABP1, which when deleted shows no phenotype but leads to an aberrant actin phenotype when over-expressed (Drubin, Miller, & Botstein 1988). In order to achieve this, SCP1 cDNA was cloned downstream of the inducible methionine promoter (sections 2.1 and 3.2.3) in both the CEN plasmid which will produce 2 copies of the protein or the high copy number 2- μ m plasmid (Fig. 3.10c), and then transformed into wild type yeast cells.

Over-expression from both of these plasmids resulted in lethality when grown on agar plates (Fig. 3.17). In order to assess in more detail possible causes of this lethality the SCP1 gene was over-expressed for shorter period in cells in liquid media, and the cells were then stained with rhodamine -phalloidin in order to visualise the actin structures (section 2.4.1). The results of this experiment (Fig. 3.18) show a clear time-dependent disruption of both actin patches and cables. Within 4 h there was a clumping of actin patches at the site of bud formation; however after 20 h where the aberrant clumps of actin could still be visualised, the cells appeared smaller and un-budded.

d.o. URA



d.o. URA, MET

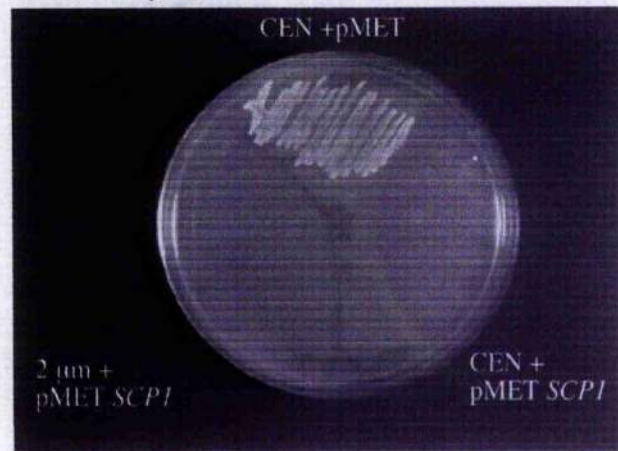
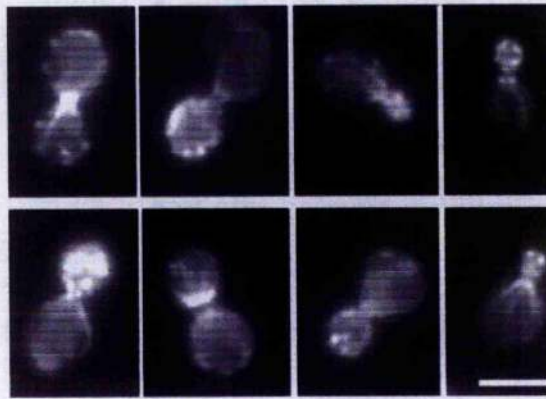


Fig. 3.17 Effects of overexpression of SCP1 gene in *S. cerevisiae*

Yeast cells containing a SCP1 gene under the control of an inducible methionine promoters on either the CEN; 2 copy or 2 μ m; multiple copy plasmid, were plated onto synthetic media lacking uracil (d.o. URA) (necessary to maintain the plasmid within the cell). Scp1p overexpression was induced by plating the cells on to synthetic media lacking uracil and methionine (d.o. URA, MET), alongside cell containing only the methionine promoter. Overexpression from either the CEN or the 2 μ m plasmid resulted in an almost complete lack of growth, whereas the cell containing just the methionine promoter appeared to grow normally.

pMET *SCP1* uninduced



pMET *SCP1* induced

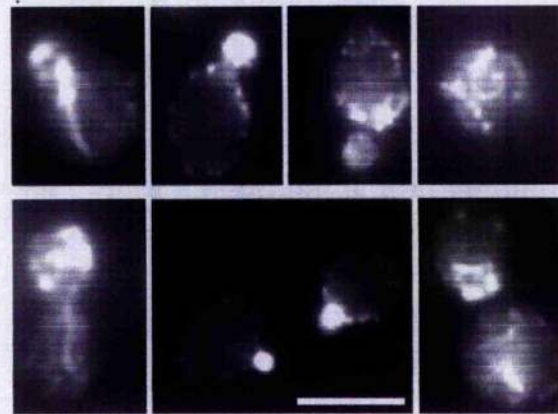


Fig. 3.18 Effect of over expression of the *SCP1* gene on *S. cerevisiae* grown in liquid media: Cells containing the plasmid responsible for overexpression of the *SCP1* gene were grown in the presence (top) and absence (bottom) of methionine. Both sets of cells were fixed and stained with rhodamine –phalloidin to detect actin, which can be seen to form both actin patches and cables in the the uninduced cells, compared to the large aggregates of actin which can be seen in cells induced to overexpress *Scp1p*. Scale bars 5 μ m.

4 Discussion

In this study it has been shown that *in vitro* Scp1p, a member of the calponin, SM22/transgelin family of proteins both binds to actin and induces the formation of tight actin bundles. Furthermore it has been shown that the carboxy terminal region of Scp1p including the CLR and the preceding sequence (but excluding the CH domain) is sufficient to bind actin. The role of Scp1p *in vivo* and has also been investigated and it has been shown that Scp1p localises to actin structures within the cell and acts with other actin-binding proteins to regulate actin dynamics.

Biochemical analysis using high and low-speed sedimentation assays together with viscosity assays has shown that Scp1p binds to actin with a K_d of $\sim 0.5 \mu\text{M}$, a figure similar to that reported for calponin which binds actin with a K_d of $1 \mu\text{M}$ (Lu, Freedman, & Chalovich 1995). Calponin and SM22/transgelin have also been reported to induce the formation of organised actin networks (Kolakowski et al. 1995; Shapland et al. 1993); here it has been shown by EM that Scp1p is able to organise actin filaments into tight bundles. At first sight this finding would appear to be at odds with the falling ball data which showed a decrease in the viscosity of a solution of F-actin, indicating that Scp1p was involved in severing of actin filaments. This apparent contradiction has also been reported in proteins such as scruin (Owen & DeRosier 1993; Sanders et al. 1996); and can be explained by the formation of very tight parallel bundles which can be easily displaced to create a free channel for the falling ball. The kinetics of polymerisation of actin in the presence of Scp1p also suggests that Scp1p can stabilise very short filaments of actin. In order for a protein to be able to bundle actin it requires either two actin-binding sites within a single polypeptide or the ability to dimerise to form two actin-binding sites. Purified Scp1p was subjected to analytical gel-filtration, and assessed to be monomeric, a finding also reported for calponin and SM22/transgelin (Shapland, Hsuan, Totty, & Lawson 1993). It has previously been shown that CH domains are not sufficient or necessary to bind actin but may serve to

SM22/transgelin have reported two putative actin-binding sites located in the CLR and the region between it and the CH domain. In the present work a 6 kDa fragment of Scp1p corresponding to the region C-terminal of the CH domain including the CLR has been identified, and shown to be sufficient to bundle actin in a low-speed sedimentation assay. Calponin family members contain a conserved serine in the first CLR (Ser¹⁷⁵ in calponin, Ser¹⁸¹ in SM22/transgelin), which has been shown to be critical for actin binding (Fu et al. 2000; Winder et al. 1993). Since Scp1p also contains this conserved residue (Ser¹⁸⁵) it is likely to function in a similar fashion. A second actin-binding site must therefore reside in the region between the CH domain and the CLR. In a similar manner to ABS1 in calponin (EL Mezgueldi et al. 1996) this site consists of a stretch of basic amino acids, which could act as a poly-cation that is able to interact with acidic actin filaments. Some doubt has been raised over the validity of the reports concerning the actin-bundling activity of SM22/transgelin (Morgan & Gangopadhyay 2001). Since bundling only occurs at low ionic strengths it is possible that it could be due to non-specific electrostatic interactions. In order to rule out this possibility all experiments on the properties of actin binding by Scp1p were carried out at a physiological salt concentration.

Using a *S. cerevisiae* strain from which the SCP1 gene had been deleted no aberrant actin phenotype could be observed, indicating that Scp1p may have a shared function with another protein. The fimbrin homologue Sac6p has previously been shown to localise to actin patches *in vivo*, and has been implicated in the regulation of actin dynamics (Drubin, Miller, & Botstein 1988). In this work it has been shown that GFP-Scp1p also localises to actin patches and the relationship between these two proteins and the actin cytoskeleton has been investigated. Deletion of SAC6 and SCP1 resulted in a strain which, although viable, had a phenotype more severe than either deletion alone. Further analysis of gene-deleted strains has revealed a sensitivity to the G-actin monomer-sequestering drug Latrunculin A in both Δ SCP1 and Δ SAC6 knockouts and an increased sensitivity in a Δ SCP1/ Δ SAC6 double knockout (Winder, Jess, & Ayscough 2003). These effects are thought to result from a decrease in the stability of actin filaments leading to an increase in G-actin turnover. It has also shown that overexpression of SCP1 causes the formation of aberrant actin structures, presumably as a consequence of a more stabilised actin cytoskeleton and a decrease in actin dynamics.

A report by Goodman et al, published at the same time as this work was completed, has also shown that Scp1p is an actin-binding protein with the ability to cross-link actin filaments into larger networks (Goodman et al. 2003). Their biochemical and genetic

analysis of Scp1p truncation mutants has validated the results in this Thesis concerning the location of the actin-binding sites and have also shown that Ser¹⁸⁵ is indeed critical for actin binding. Goodman *et al.* have also shown localisation of Scp1p to actin patches in *S. cerevisiae* and have reported a functional relationship between Scp1p and the fimbrin homologue Sac6p in the control of the actin cytoskeleton.

More recently Scp1p has been implicated as being involved in the regulation of the actin cytoskeleton and playing a role in cell ageing (Gourlay *et al.* 2004). Δ Scp1p strains were shown to be viable for longer than wild type yeast, and strains overexpressing Scp1p exhibited a reduction in the length of time cells were viable and an increased level of reactive oxygen species ROS in the cell. These findings suggest a link between the stability of actin filaments and mitochondrial integrity in regulating cell death.

Further work is necessary to characterise fully the regions of Scp1p which interact with actin, and is likely to include engineering a larger panel of mutant proteins, with emphasis on the regions between the CH domain and the CLR/CLIK23 repeat, and the residues surrounding Ser¹⁸⁵. Further work with genetically modified *S. cerevisiae* strains is required to understand more completely the role of Scp1p *in vivo* and further characterise its relationship with Sac6p in the organisation of the actin cytoskeleton.

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